EFFECTS OF ETHANOL ON *OENOCOCCUS OENI*:

STRESS RESPONSE, ADAPTATION AND PERFORMANCE

Maria Graça da Silveira

Promotor: **Prof. Dr. Ir. F. M. Rombouts**
Hoogleraar in de Levensmiddelenhygiëne en -microbiologie,
Wageningen Universiteit

Co-Promotor: **Dr. T. Abee**
Universitair hoofddocent
Levensmiddelenhygiëne en -microbiologie,
Wageningen Universiteit

Leden van de Promotiecommissie:

Prof. Dr. M. C. Loureiro-Dias
Instituto Superior de Agronomia, Portugal

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Katholieke Universiteit Nijmegen

Dr. J. Hugenholtz
NIZO, Ede

Dr. E. A. Golovina
Wageningen Universiteit

Prof. Dr. Ir. A. J. M. Stams
Wageningen Universiteit

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Proefschrift

Ter verkrijging van de graad van doctor
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PROPOSITIONS

1. A pure culture of *Oenococcus oeni* is not a homogeneous population but a heterogeneous collection of individual cells.

   This thesis

2. Ethanol-adapted *Oenococcus oeni* cells display an increased malolactic activity, making them highly effective in deacidification of wine.

   This thesis

3. "What a trifling difference must often determine which shall survive, and which perish" (Charles Darwin). Like in the microbe world, difficult challenges are needed to reveal those trifling differences in human beings.

   In letter to Asa Gray, September 5th, 1857

4. "I haven't failed. I've found 10,000 ways that won't work." (Thomas Edison). As a scientist we should never find truth disappointing; usually the most frustrating results are those that trigger the most interesting findings.

   In Inventing the Future by M. F. Delans and D. E. Sloane

5. "There is nothing in a caterpillar that tells you it's going to be a butterfly." (Buckminster Fuller). This metamorphosis describes perfectly the process of writing a thesis out of a pool of experimental data.

   In Buckminster Fuller's universe: His life and work by L. S. Sieden and N. Cousins

6. To achieve the so desired world peace, we should understand urgently that "happiness is not a destination but a way of traveling". (Roy M. Goodman)

Propositions belonging to the thesis

"Effects of ethanol on *Oenococcus oeni*: Stress response, adaptation and performance"

Maria Graça da Silveira

Wageningen, 29 October 2003

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

1.1. Abstract

This chapter provides an overview of malolactic fermentation in wine and describes the main difficulties concerning the development of reliable ready-to-use starter cultures in the production of wine. Aspects concerning the metabolism and stress response in *Oenococcus oeni* are briefly discussed. Microbial stress response and adaptation to ethanol are presented, with special focus on the interaction of ethanol with the cytoplasmic membrane. Finally, the emerging awareness of heterogeneity among bacterial cells and its impact on stress tolerance of individual cells and subpopulations is discussed.

1.1. Malolactic fermentation

Lactic acid bacteria (LAB) are responsible for the decarboxylation of malic acid to lactic acid, during vinification. This was shown by Peynaud (71) in the first published results on the LAB microflora of grape must and wines. After the work of Ribereau-Gayon et al. (75), this second phase of vinification, called malolactic fermentation (MLF), has been intensively studied worldwide by numerous researchers (for a review see [53]). *Oenococcus oeni* formerly called *Leuconostoc oenos* (24) is recognized as the principal LAB responsible for the MLF, since it is able to grow in the hostile wine environment (29, 96).

In cold wine regions, MLF is deemed essential in order to decrease the excess acidity resulting from the high content of malic acid in grapes grown in such climates. This decrease in acidity improves the wine "mouthfeel" which becomes "softer". Another benefit of MLF is to improve microbiological stability of the wine (57). During its activity in wine, *O. oeni* cells also ferment residual sugars, hexoses and pentoses (Fig. 1-2) left by yeasts (76). Other known (e.g. citric acid) and unknown substrates are metabolized contributing to the flavour complexity of wine.

The biological advantage of MLF has been a matter of discussion among enologists for years. The decarboxylation of L-malic acid is a one step reaction catalyzed by a single enzyme, called malolactic enzyme (MLE), which is NAD^+ and Mn^{2+} dependent (Fig. 1-1). The lack of a pyruvate intermediate (14) and the fact that direct H^+ or Na^+ extrusion involving membrane-bound decarboxylases does not occur (25), prompted researchers to report that MLF serves a non-energy-yielding function. It was suggested that MLF improved growth by creating a favorable pH that stimulates utilization of other substrates (15, 72). It is now well accepted that the MLF is a proton motive force- generating process that occurs in various LAB (55, 68, 74, 80). In *O. oeni* the proton motive force (pmf) is generated during MLF as a consequence of the electrogenic transport of monoprotonated malate and concomitant consumption of a proton in the cytoplasm during its decarboxylation (74, 81). The pmf generated is of sufficient magnitude to drive ATP synthesis by the H^+-ATPase (21, 39, 80).

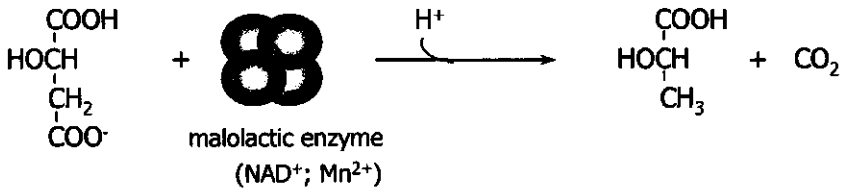

Figure 1-1 Schematic representation of the malolactic reaction in whole cells of *O. oeni* at wine pH.

MLF may occur spontaneously in wine as a result of the growth of an indigenous flora of LAB. However, MLF depends upon the growth of LAB in the wine as a batch culture and is strongly influenced by environmental conditions, so that the naturally developing process is often delayed or fails, and consequently introduces significant and inconvenient delays into the overall process of vinification (97). The time-honored methodology, where the winemaker relies on the bacteria resident in barrels in which a desirable MLF has previously occurred, call for a wine storage in conditions that prevent the development of undesire d fermentations, i.e. cool and with added sulfur dioxide, which may itself delay the eventual

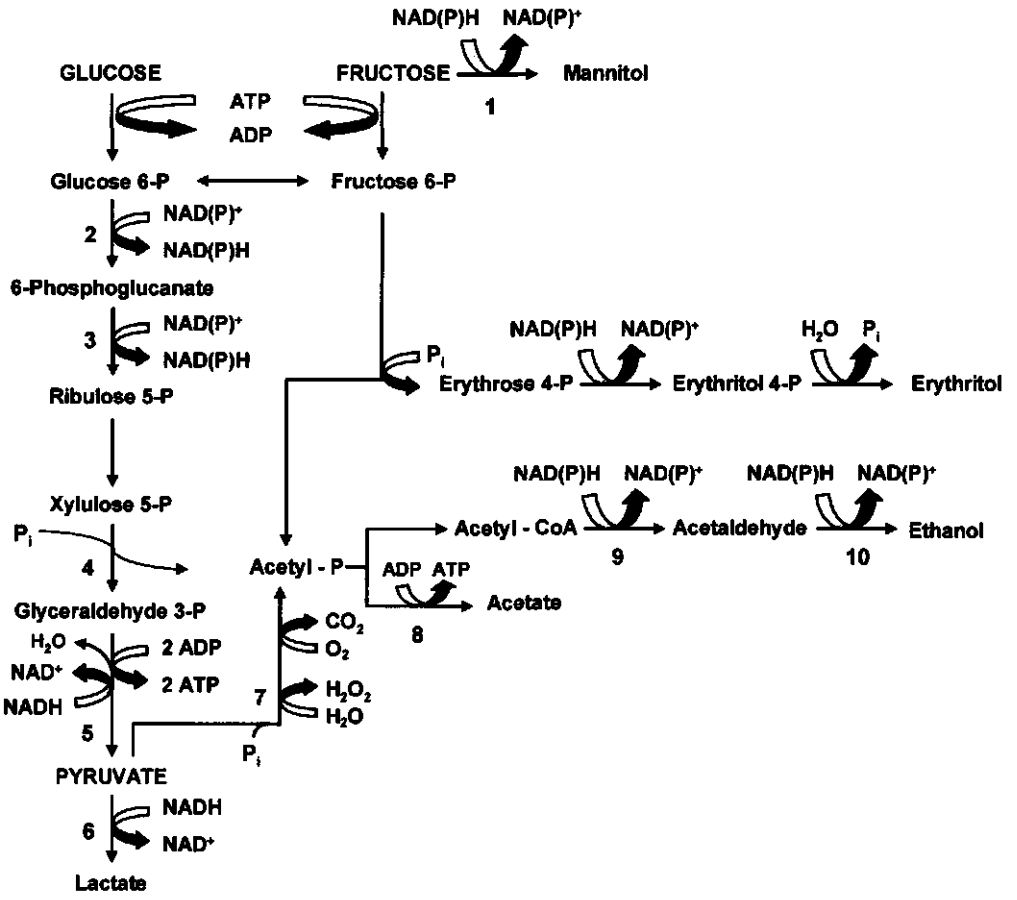

Figure 1-2 Schematic representation of carbon and energy flows through the central metabolic pathways of *O. oeni*. Numbers represent: 1, mannitol dehydrogenase; 2, glucose 6-phosphate dehydrogenase; 3, phosphogluconate dehydrogenase; 4, xylulose 5-phosphate phosphoketolase; 5, glyceraldehyde 3-P dehydrogenase; 6, lactate dehydrogenase; 7, pyruvate oxidase; 8, phosphate acetyltransferase; 9, acetaldehyde dehydrogenase; 10, alcohol dehydrogenase.

MLF for several months after alcoholic fermentation (50). Spontaneous fermentation has become even more unpredictable as winery hygiene has improved, e.g. as a result of the replacement of wooden casks with stainless steel tanks for fermentation of red wines (67). This fact encouraged the development of technology for the induction of MLF by inoculation of industrial starter cultures (28, 67). However, inoculation of *O. oeni* starter cultures directly into wine leads to significant cell mortality and, consequently failure of MLF. Indeed, starter cultures need one or more steps of reactivation and adaptation to wine, in order to enhance the survival of the bacteria after inoculation into wine (51, 64, 65, 67). These steps, which are very time consuming, require microbiological expertise in order to reduce the risk of contamination with other bacteria, and this has limited the practical application of starter cultures. An understanding of the mechanisms involved in wine toxicity and tolerance in *O. oeni* is required for the construction of such tolerant strains for direct inoculation of wine. Thus, advances in commercial starter production are dependent on fundamental research on physiology of *O. oeni*.

1.2. Wine as a stressful environment

Basic knowledge of MLF has increased considerably from the moment enologists recognized the importance of this secondary fermentation in wine. However, many gaps still exist and one of the most evident ones concerns the adaptation of these bacteria to such a harsh medium as wine including low pH, toxic substances produced by yeast metabolism during alcoholic fermentation e.g. 10-12% ethanol and fatty acids such as octanoic acid (54, 11). Moreover, red wines contain large amounts of phenolic compounds (18), present in grape skins and seeds, which are extracted during winemaking. The addition of SO_2 to grape must in the beginning of the vinification process is a current practice (38) to control oxidation reactions and restrict the growth of undesirable microorganisms, mainly acetic acid bacteria (6). This stress condition may also affect the performance of *O. oeni* in wine (13, 35).

Until now, many studies on stress response in *O. oeni* have been concerned with the effect of several factors on MLF and growth of *O. oeni* (11, 49, 73, 82, 97). However, knowledge about the physiological mechanisms involved in stress response and adaptation in *O. oeni* is limited. In this respect, much attention has been devoted to aspects regarding acid tolerance (26, 31, 63, 90). Three mechanisms appear to play an important role: (i) MLF which is involved in proton motive force (pmf) generation and in the maintenance of internal pH by proton consumption during L-malate decarboxylation (80), (ii) the activation of proton-extruding ATPase (26), and (iii) stress protein synthesis as part of a typical acid tolerance response (ATR) (34). Pmf becomes important as the external pH decreases and the concentration of essential nutrients that are transported by secondary transport systems becomes low (80), conditions that are generally found in wine. This is in line with the fact that the first two mechanisms claim the maintenance of pmf generating capacity to be a crucial feature in the performance of *O. oeni* in wine. Wine has a complex composition, so a central composite design is an effective way to study the interaction among several factors (32). Vaillant et al. (92) studied the effect of 11 physico-chemical parameters on

MLF in three strains of *O. oeni* and showed that ethanol had the largest inhibitory effect. Since ethanol acts as a disordering agent of the *O. oeni* cytoplasmic membrane, leading to leakage of intracellular material absorbing at 260 nm (chapter2) and promoting pmf dissipation, the superimposed effect of ethanol over all other stress conditions as observed by Vaillant et al (92) is understandable.

1.3. Effect of ethanol

The accumulation of ethanol as a product of alcoholic fermentation carried out by yeast represents an adverse environmental change for *O. oeni* that has to perform MLF under such conditions. Ethanol changes the physical characteristics of the environment of a cell (30) and may alter the way in which a cell interacts with its environment. In the beginning of the 80's a lot of work was done in this field and a number of "inhibitory" mechanisms have been proposed, however, the exact range of biochemical and physiological processes affected by ethanol is mostly undefined. Therefore it is very well possible that the investment in ethanol research was misdirected due to this deficiency in fundamental understanding.

Since, the cytoplasmic membrane is the primary site through which the cell maintains contact with its surrounding, it can be expected to be the primary site of the cellular defense against ethanol. Although, biological membranes provide the structural framework that separates cells interior from the environment, they are not simply passive barriers, on the contrary, cytoplasmic membranes can be indeed regarded as extremely dynamic structures involving specific lipid-lipid and lipid-protein interactions. Domains formed by integral and intrinsic proteins divide the membrane into regions differing in lipid and protein composition, so that the free energy of the system is minimized. When cells are suddenly exposed to ethanol the toxic effect is generally attributed to the preferential partitioning of ethanol to the hydrophobic environment of the lipid bilayer. However, this is hard to reconcile with the polar and hydrogen-bonding properties of ethanol and with its full miscibility with water. Jones (44) in an extensive review of biological principals of ethanol toxicity supports the theory that membrane-located effects of ethanol are more likely to be due to dielectric disruption of the aqueous phase, to competition with water for polar membrane sites, and to selective location within the polar region of membrane surfaces or proteins.

The physico-chemical state of the membrane, often called the "membrane fluidity", is determined by bulk lipids and quantitatively expressed as the inverse of viscosity. Fluidity is a somewhat ambiguous term as it combines the effect of both lipid dynamics and acyl chain order. Dynamic processes include lateral and rotational diffusion of the lipid molecules, and also rotation around single carbon-carbon bonds. Acyl chain order or lipid packing refers to the average orientation of each carbon along the chain (88). The motion of spin-labels used to assess fluidity (see also chapter 4) reflects both dynamics and molecular order. The physiological relevance of fluidity is evident from the adaptation of various organisms to environmental stresses. Ethanol tolerance has been associated with high plasma membrane fluidity both in yeast (1, 2, 84) and bacteria (5, 20, 22, 40). The fluidization response can be interpreted on the basis of the hypothesis of "homeoviscous adaptation" (85) as a counteraction to the physico-chemical effect of ethanol

on membranes (42). This model, whilst being widely reported, is apparently not universally applicable to all organisms. Exceptions have been reported for *B. subtilis* (77), *E. coli* (27), and *O. oeni* cells (chapter 4 in this thesis), in which cytoplasmic membranes isolated from cells grown in the presence of ethanol were more rigid than those from the control cells.

Eukaryotic cells modulate membrane fluidity by changing the phospholipids/cholesterol ratio. However, bacteria do not contain cholesterol and the ability to adjust their membrane fluidity is to a large extent dependent on changes in fatty acid (FA) composition (for a review see [79]). The ethanol-induced changes in membrane fatty acid unsaturated/saturated ratio are widely recognized, and it is claimed that an increase in membrane unsaturation is the mechanism leading to ethanol tolerance both in yeast and bacteria (17, 27, 40, 42, 62, 89). Caution is required, however in what dictates the requirement for unsaturated FA in the acquisition of ethanol resistance. Ethanol has been shown to inhibit the biosynthesis of saturated fatty acids in bacteria (10) and conceivably in the presence of ethanol cells display higher amounts of unsaturated FA. In a comprehensive study of adaptation of *Pseudomonas putida* S12 to ethanol (37) it was observed that ethanol promotes a strong increase of *trans* unsaturated FA and at the same time decreases the degree of saturation of the membrane FA. The isomerization of *cis* to *trans* unsaturated FA compensates for the ethanol-induced fluidizing effect, yet, the decrease in the degree of saturation has an antagonistic effect. These observations strongly support the hypothesis that the decrease in the degree of saturation induced by ethanol is a compensatory effect rather than an adaptive mechanism of the cells.

1.4. Membranes as stress sensors

The stress-sensing systems leading to the cellular heat shock response (HSR) and the mechanism responsible for desensitizing this response in stress acclimatized cells are largely unknown. The classical view of the HSR is that stressing agents cause the accumulation of aberrant or partially denatured proteins in the cell with a resultant induction of the genes responsible for HS protein production (reviewed in [69] and [86]). However, a number of independent observations support the hypothesis that the stress-sensing mechanisms are intimately associated with membrane structure and function, e.g. (i) the plasma membrane is a very sensitive monitor of environmental induced changes, (ii) the activity of integral membrane proteins is sensitive to the lipid composition, the lipid bilayer dynamics and its physico-chemical state (59).

Chatterjee et al. (16) demonstrated that the thermal inducibility of a heat shock (HS) sensitive reporter gene is closely correlated with the percentage of unsaturated fatty acids present in yeast cells. The authors observed that yeast cells supplemented with high levels of unsaturated fatty acids displayed an increase of up to 9°C in the optimal activation temperature for the HS response (HSR). These results suggest that the transient nature of the HSR may be a manifestation of a lipid-mediated desensitization of the heat stress signal transduction mechanism. Further evidence comes from a study carried out by Carratu *et al.* (12) showing that membrane lipid composition affects the set point of the temperature of HSR

in yeast. Since, ethanol is known to affect membrane composition (see [44]) we can expect ethanol stress to be transduced into a cellular signal at the level of the membrane. Actually, ethanol was found to lower the threshold temperature for the maximal activation of HSR in *S. cerevisiae* (23).

The involvement of membrane composition in the stress-sensing pathway is not restricted to yeast. An unbalanced membrane phospholipid composition of *E. coli* induced a phospholipid-specific stress signal to which certain regulatory genes responded positively or negatively according to their intrinsic mechanisms (43). This observation allows us to speculate that changes in the lipid species that surround the transmembrane segments of the sensing domain of the two-component system modulate the activity of the histidine kinase (Fig. 1-3). Thus, in response to a composition change, the signaling pathway is switched on via a phosphorylation event.

In a very elegant assay, Horvath et al (41) demonstrated that in *Synechocystis* the temperature at which maximal activation of a number of HSR genes occurs decreased when the fluidity of the thylakoids was increased. Thus, the physical state of the membrane itself is proven to be involved in the transduction of heat stress into a biological signal. Changes in membrane order seem to be important for regulation of gene expression also in the yeast *PKC1* gene (47). This gene, whose product is implicated in the regulation of a MAP-kinase pathway, could be activated when cells detected a stretch in their plasma membranes after thermal stress. Moreover, in *Lactococcus lactis* changes in the transmembrane osmotic gradient are transmitted to the osmoregulated ABC transport system via distortions in the membrane bilayer, which is consistent with a role for membrane strain (or curvature stress) in the activation of the sensing mechanism (93).

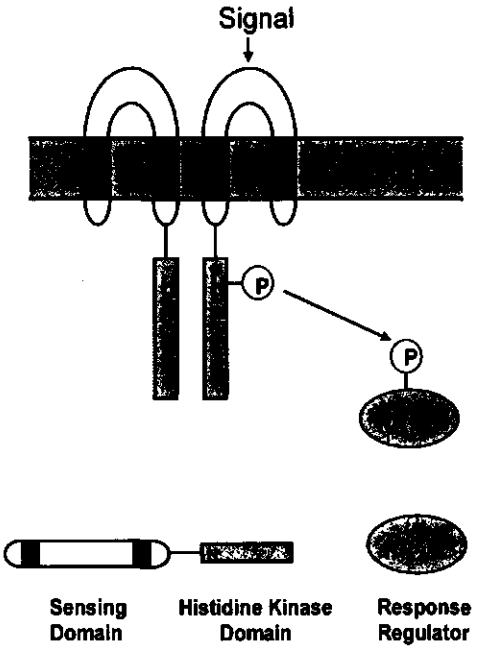

Figure 1-3 Two-component signalling mechanisms. Prokaryotes signalling pathway is structured around two proteins. The first component is a transmembrane ATP-dependent histidine protein kinase (HK), composed of a periplasmic sensor domain and a cytoplasmic histidine kinase domain that catalyse ATP-dependent autophosphorilation. The second component of the system, a cytoplasmic response regulator protein (RR), is activated by the HK. The sensor domain detects stimuli and modulates the activity of the cytoplasmic kinase domain, thus regulating the level of phosphorylation of the RR. Posphorylation covalently modifies the RR, which results in its activation and generation of the output response of the signalling pathway (87).

1.5. Measuring heterogeneity

Information of individual cells in a microbial culture was restricted, until few years ago, to the determination of colony forming units in plate. This common microbiological practice relied on the assumption of uniformity within a bacterial population that constitutes a colony, which was supported by the results obtained from classical techniques that involved bulk measurements. However, when one considers survival of organisms, the variation between individual cells is unmasked since not all cells survive equally well (45). When cells are exposed to a stress, the decline of the population frequently follows first order kinetics, which is inconsistent with uniformity of susceptibility (95).

Next to the obvious heterogeneity in a mixed natural populations microbial heterogeneity arises from four principal sources: first, genotypic through mutation; second, phenotypic via progression through the cell cycle (19); third, phenotypic by changes in the exact local environment and its history (60) and fourth, arises from oscillatory intracellular dynamics, e.g. ultradian clock-controlled properties (56). Booth (7) suggests that the transient super abundance of specific proteins that counter stress is responsible for the extreme survival properties of a few cells. Four core systems in the cell are capable of transiently altering protein balance: gene duplications, altered initiation of transcription, proteolysis and competition for ribosomes. In theory, the cell could develop a tighter control mechanism designed to match supply to demand. However, cells would rule out heterogeneity and consequently sacrifice adaptability, which is one of the most significant traits possessed by bacteria. The advantage to the species of heterogeneity within the population is that no matter what stress is encountered by the organism some cells will survive and can potentially become the new colonists of a "purged niche" or of a newly encountered environment.

The mainstream of work in microbial physiology is typically done with nonsynchronised batch and continuous cultures. Let us consider a study of biotechnological application in which one might wish to establish the relationship between one or more metabolic properties of interest and the loss of fermenting capacity. We may, for instance, monitor the activity of a specific metabolic enzyme of the bacterial culture. However, when one studies the extent to which the activity of an enzyme determines the fermentation capacity of a population, one has to consider the possibility that the activity of that enzyme is distributed heterogeneously among individual cells (46, 48). Thus a technique, which enables measurements to be made in single cells, provides incomparably more useful information than do traditional biochemical assays, which report average values of large populations of cells. Flow cytometry (FCM) is the obvious tool for single-cell analysis of a large number of cells. It is an optical, purely quantitative, technique, which measures scattered light and fluorescence properties of individual cells (83). Advantages of using fluorescence techniques in combination with FCM are a high sensitivity (i.e., the number of molecules needed for detection is low), a high time resolution (approx. 10^{-6} s), and the possibility of a view into living cells without disturbing the cellular organization.

It has been very elegantly demonstrated that labeling cells with fluorescent probes allows specific assets of cell physiology to be studied (for reviews see [8]). A large number of fluorescent dyes has been described, for instance, as vital stains (36). A classical example is the esterified pre-fluorochrome carboxyfluorescein diacetate (cFDA) that is converted to carboxyfluorescein (cF) by functional cytoplasmic enzymes i.e. esterases. cF is negatively charged at physiological pH, and consequently will accumulate inside cells with an intact cytoplasmic membrane (78). Indeed, demonstration of esterase activity provides an indication of metabolic activity, showing the capacity of a cell to have synthesized this enzyme in the past and its ability to maintain it in an active form. But, the cFDA cleaving reaction giving rise to fluorescence is typically not energy dependent. However, the ability to transport molecules across the membrane or regulation of pH requires energy metabolism. Thus cF extrusion, more than cF labeling capacity, can provide a highly sensitive indicator of cell stress as we demonstrate in chapter 3 of this thesis. Therefore, assays should be designed to give information about the selected parameters being investigated rather than to give an estimate of cell viability.

1.6. Use of Multi-staining Flow Cytometry in Food Industry

Rapid assessment of microbial presence and viability in food products is of paramount importance for the food industry, daily compelled with the production and control of beneficial and undesired (spoilage, pathogenic) microorganisms, respectively. So, analytical techniques decoupled from post-sampling growth are desirable to allow on site detection of microorganisms in foods, which may enable food processors to achieve real-time data useful for HACCP system implementation. The FCM analysis fulfils these requests and has been proven to be a reliable technique to monitor total bacterial counts in food (33, 52, 70). The use of specific fluorescent dyes in combination with FCM improved the detection of foodborne microorganisms, given that food samples have a very high background (61, 91, 94). However, viability of food pathogens, defined by reproductive growth on agar plates, often cannot be measured due to irreversible DNA damage, fastidious growth requirements or extremely slow growth. Multi-colour flow cytometric analysis allows differentiation of stages far beyond the classical definition of viability (4, 66), namely, detection of metabolic activity that provides presumptive evidence of reproductive growth. In food industry, this approach can represent an interesting alternative, since metabolism even in the absence of growth may produce undesirable effects such as food spoilage and/or accumulation of toxins.

Given the speed of analysis and the diversity of cell physiological characteristics that can be measured, flow cytometry can be applied for online-monitoring providing detailed information about fermentation processes. Thus, it represents a sophisticated tool that allows direct regulation of the process conditions in order to optimize the production. The potential use of FCM in oenological industry has become more and more a real possibility. FCM as been shown to be a good method to enumerate dry wine yeasts (9) as well as in predicting the performance of these yeasts after rehydration (3). More recently, Malacrino et al. (58) used FCM to count simultaneously yeasts and malolactic bacteria in wine. They found

a good correlation between viable counts determined by FCM and the number of colony-forming units (cfu) observed in plate counts. This is of special importance if we consider that *O. oeni*, the principal malolactic bacteria of wine, takes 1 week to form visible colonies on plates. Moreover, the presence of a large number of injured-cells after inoculation of *O. oeni* starter cultures in wine or at any time throughout the course of MLF has an obvious detrimental effect on the efficiency of malic acid degradation. In chapter 2 of this thesis we present a rapid method to assess membrane integrity of *O. oeni* cells, of major interest to the starter culture industries as an indicator of the physiological state of the individual *O. oeni* cells. In chapter 3 we provide a rapid method to assess the bioenergetics of individual *O. oeni* cells, which represents a promising technique to the wine industry allowing online monitoring of malolactic activity at the single cell level.

1.7. Scope of this thesis

Although much attention has been devoted to the effect of several stresses, including ethanol, on the malolactic activity and growth of *O. oeni*, aspects regarding mechanisms involved in ethanol adaptation have been poorly considered. The objective of the present work is to provide data that will contribute to understanding the mechanisms involved in ethanol-stress and adaptation in *O. oeni*, necessary for the design of ethanol-tolerant (or more generally wine-tolerant) starter cultures for direct inoculation in wine.

In the scope of this work *O. oeni* viability is not considered since starter cultures should be seen as "reservoirs" that maintain malolactic enzyme in optimal conditions to perform MLF. Because of the optimum pH of the enzyme (around 5.8), the need for cofactors (Mn^{2+}, NAD^{+}), and the inhibitory effects of many wine components (carboxylic acids, polyphenols), the protein must be protected from the medium by the cell membrane. Thus, the malolactic activity of the cells is strictly dependent on the integrity of the plasma membrane.

In **Chapter 2** the effect of ethanol on the cytoplasmic membrane integrity is described. Membrane integrity of ethanol-stressed and adapted cells was assessed both at the population and single cell level by Multiparameter FCM.

In **Chapter 3** the effect of ethanol on organization of cytoplasmic membrane of *O. oeni* cells was studied in further detail by electron spin resonance spectroscopy (ESR). The role of membrane composition changes in the acquired tolerance to ethanol was also investigated.

In **Chapter 4** the performance of *O. oeni* under ethanol-stress conditions is outlined. In order to assign bulk activities measured by classical methods to the very active cells that are effectively responsible for the observations, we used cFDA in combination with FCM to discriminate between cells showing no MLF activity and highly MLF active cells. Our approach is outlined in figure 1-4.

In **Chapter 5** we looked for an active ethanol adaptation response of *O. oeni* at different cellular levels using a proteomics approach. To understand the physiological relevance of the site-specific location

of proteins involved in ethanol adaptation, cytoplasmic, membrane-associated and integral-membrane proteins were investigated.

In **Chapter 6** an overview of the effect of ethanol stress and adaptation on the performance of *O. oeni* and the mechanisms involved in ethanol-stress and adaptation are given. The fundamental knowledge achieved under the topic of this thesis is also generally discussed in a context of practical applications.

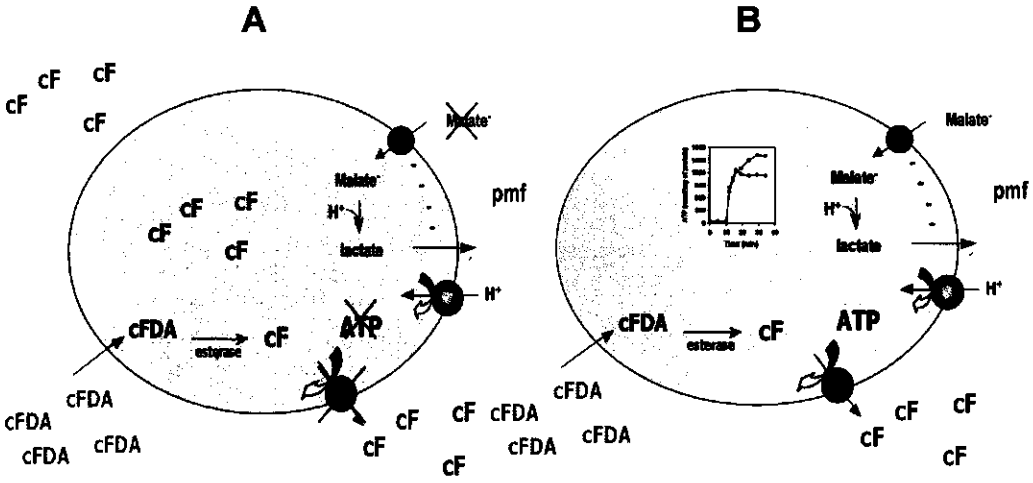

Figure 1-4 Schematic figure of cF labeling and efflux in *O. oeni* cells. (A) Leakage of cF by passive diffusion is used as an indicator of membrane integrity in de-energized cells. (B) Active extrusion of cF is used as an indicator of metabolic activity in cells performing MLF.

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2. FLOW CYTOMETRIC ASSESSMENT OF MEMBRANE INTEGRITY OF ETHANOL-STRESSED CELLS OF *OENOCOCCUS OENI*

2.1. Abstract

The practical application of commercial malolactic starter cultures of *Oenococcus oeni* surviving direct inoculation in wine requires insight into mechanisms involved in ethanol toxicity and tolerance in this organism. Exposure to ethanol resulted in an increase of the permeability of the cytoplasmic membrane enhancing passive proton influx and concomitant loss of intracellular material (absorbing at 260 nm). Cells grown in the presence of 8% (vol/vol) ethanol revealed adaptation to ethanol stress, since these cells showed higher retention of compounds absorbing at 260 nm. Moreover, for concentrations higher than 10 % (vol/vol) lower rates of passive proton influx were observed in these ethanol-adapted cells, especially at pH 3.5. The effect of ethanol on *O. oeni* cells was studied using the ability to retain efficiently carboxyfluorescein (cF) as an indicator of membrane integrity and enzyme activity, and the uptake of propidium iodide (PI) to assess membrane damage. Flow cytometric (FCM) analysis of both ethanol-adapted and non-adapted cells with a mixture of the two fluorescent dyes cF and PI, revealed three main sub-populations of cells i.e. cF-stained, intact cells; cF and PI-stained, permeable cells; and PI-stained, damaged cells. The sub-population of *O. oeni* cells that maintained their membrane integrity, i.e. cells stained only with cF, was 3 times larger in the population grown in presence of ethanol, reflecting the protective effect of ethanol adaptation. This information is of major importance in studies of microbial fermentations in order to assign bulk activities measured by classical methods to the very active cells that are effectively responsible for the observations.

Da Silveira, M. G., San Romão, M. V. Loureiro-Dias, M. C., Rombouts, F. M. and T. Abee. 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. Appl. Environ. Microbiol. **68**:6087-6093.

2.2. Introduction

The control of activity of lactic acid bacteria that carry out malolactic fermentation (MLF) is an important feature of the technology of modern commercial wine production (26). *Oenococcus oeni* is recognized as the principal microorganism responsible for the MLF (22), under stress conditions as those prevailing in wine. MLF consists of the decarboxylation of L-malic acid to L-lactic acid, which decreases the total acidity and improves the stability and quality of wine (25). The physiological benefits of MLF to the bacteria have been a matter of discussion for the last few years, but it is now well-accepted that the malolactic activity generates an electrochemical gradient across the cytoplasmic membrane as a consequence of the electrogenic transport of monoprotonated malate and concomitant consumption of a proton in the cytoplasm during its decarboxylation (34, 39). The proton motive force (pmf) generated is of sufficient magnitude to drive ATP synthesis by the H^+-ATPase (6, 17, 40).

Inoculation of *O. oeni* starter cultures directly into wine leads to significant cell mortality (4) and, consequently failure of MLF. Actually, one or more steps of reactivation and adaptation of starter cultures to wine conditions are required, in order to enhance the survival of the bacteria when inoculated into wine (22). The ethanol and acid resistance of *O. oeni* are considered to be crucial for its survival in wine. Three mechanisms appear to be involved in acid tolerance of *O. oeni*: (i) activation of MLF as a proton motive force generating process (40), (ii) stress protein synthesis (14), and (iii) activation of membrane-bound H^+-ATPase (11). The ethanol toxicity is generally attributed to the preferential partitioning of ethanol in the hydrophobic environment of lipid bilayers, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes (29). It has been reported that membrane disordering resulting from ethanol exposure leads to a leakage of intracellular compounds, including enzymatic cofactors and ions essential for cell growth and fermentation (33, 41), as well as the dissipation of the electrochemical gradient across the cytoplasmic membrane (24, 29).

The pre-adaptation of starter cultures is very time consuming and requires microbiological expertise. Therefore, the practical application of commercial malolactic starter culture has been limited. The development of new industrial starters surviving direct inoculation represents a large benefit for wine technology (31). It is therefore essential to enhance the understanding of the mechanisms involved in ethanol toxicity and tolerance in *O. oeni*. It has been suggested that the cytoplasmic membrane may be the target for the physiological events inducing better survival of lactic acid bacteria in wine, e. g., by modifying their fatty acid composition (9, 13, 23).

The purpose of this work is to study the effect of ethanol on the membrane integrity of *O. oeni*. Ethanol-induced leakage of UV-absorbing compounds has been proven to be a valuable technique for monitoring ethanol-resistance of microorganisms e. g. of yeast (41, 42, 30, 25). In addition, since the pH of wine is very low (e. g. lower than 3.5) the passive influx of protons is an important parameter to be taken

into account. Both techniques involve bulk measurements and assume that all the cells will contribute equally to the global performance of MLF. The use of fluorescent probes in combination with flow cytometry (FCM) allows the discrimination of *O. oeni* ethanol-stressed cells in different physiological states (7). i.e. different sub-populations with respect to their ethanol tolerance can be readily identified. For that we use simultaneously a permeant (carboxyfluorescein diacetate) and an impermeant (propidium iodide) probe to assess membrane permeability and integrity of stressed *O. oeni*. Cells with intact membranes are impermeable to charged fluorescent dyes such as propidium iodide (PI). However, if membrane integrity is lost, PI can enter the cell and by binding to the nucleic acid, the cells become fluorescent. The esterified pre-fluorochrome carboxyfluorescein diacetate (cFDA) is converted to carboxyfluorescein (cF) by functional cytoplasmic enzymes i.e. esterases. cF is negatively charged at physiological pH, and consequently will accumulate inside cells with an intact cytoplasmic membrane. Thus, cF-stained cells have esterase activity and an intact membrane (16, 35). These measurements can often be complicated due to extrusion pumps, which at the same time are powerful indicators of functioning cell metabolism (1, 2). In our studies we use de-energized cells excluding the interference of metabolic activity, allowing the assessment of membrane integrity by dye retention (cF) and/or dye exclusion (PI). Moreover, monitoring the leakage from cells previously loaded with a foreign molecule (cF), allows permeability to be studied without the superimposed effect of the molecule size. Multiparameter FCM analysis of both ethanol-adapted and non-adapted *O. oeni* cells allows assessment of population heterogeneity, which may provide tools for optimization of MLF in wine.

2.3. Materials and Methods

Bacterial strain and growth conditions. *Oenococcus oeni* GM (Microlife Technics, Sarasota, Fl.) was cultured at 30°C in FT80 medium (pH 4.5) (5) modified by the omission of Tween 80, containing 10 g of DL-malic acid per liter. Glucose and fructose were autoclaved separately and added to the medium just before inoculation, at a final concentration of 2 and 8 g per liter, respectively. Early stationary phase cultures were diluted 100-fold in fresh medium, incubated for 24 h, and then used to obtain 1% inoculated cultures. In the adaptation experiments the final culture medium was supplemented with 8% (vol/vol) ethanol.

Leakage of compounds absorbing at 260 nm. The method adopted is described by Salgueiro *et al* (1988), with some modifications. Cells were harvested at the end of the growth phase (OD_{600}=0.4) by centrifugation at 6160 x *g* at 4°C for 10 min, and washed twice in potassium phosphate buffer (50 mM, pH 5.2). The cell pellet and cell suspension prepared in the same buffer at a concentration of 50 mg/ml (dry weight) were kept on ice. Cell suspension (80 µl) was placed in an Eppendorf tube, incubated at 30°C for 30 seconds and 20 µl of ethanol solution at 30□C was added. Ethanol solutions were prepared from 99% (vol/vol) ethanol in phosphate buffer in order to obtain final concentrations of 0, 8, 12 and 16% (vol/vol). Immediately after ethanol addition the reaction was stopped by dilution with 1.4 ml of the same buffer at

room temperature and samples were centrifuged for 6 minutes at 8500xg. The supernatants were removed and filtered through cellulose filters (Nucleopor) with a porosity of 0.22 μm. The total amount of compounds absorbing at 260nm was measured in a Spectrophotometer Beckman DU-70 and expressed in nmol of NAD^+ (25). Similar experiments were performed with cells pre-cultured in the same medium with 8% (vol/vol) ethanol.

Proton influx. Cells were harvested at the end of the exponential growth phase (OD_{600}=0.4) by centrifugation at 6160 x *g* at 4°C for 10 min, and washed twice in phosphate buffer (2 mM, pH 7.0). The cell suspension was prepared in the same buffer at a concentration of 30 mg/ml and it was kept on ice. Proton movements were measured at 30°C with a standard pH meter, Radiometer PHM62, connected to a recorder. In a water-jacketed cell with a volume of 10 ml, 0.3 ml of a cell suspension, distilled water and ethanol were mixed to a final volume of 3 ml. By addition of HCl (100 mM) the pH was rapidly adjusted to 3.5 or 4.5. Subsequent pH changes were registered during a time interval in which a linear pH variation was observed. At the end of each experiment, the signal was calibrated using a solution of HCl (10 mM). The rate of proton influx was expressed as the rate of decrease of the concentration of extracellular protons, according to Leão and van Uden (1984). Similar experiments were performed with cells pre-cultured in the presence of 8% (vol/vol) ethanol.

Loading of cells with carboxyfluorescein (cF). Cells were harvested at the end of the exponential growth phase (OD_{600}≈0.4) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 20. The cells were de-energized with 2-deoxyglucose (at a final concentration of 2 mM), by incubation at room temperature during 30 min. The cells were washed and re-suspended in 50 mM KPi buffer (pH 7.0) to an OD_{600} of 20 for fluorimetrical analyses or to an OD_{600} of 5 for flow cytometric analyses. A stock solution of 2.3 mg of 5(6)-carboxyfluorescein diacetate (cFDA) (Molecular probes, Eugene, Oregon) per ml was prepared in acetone and stored at -20°C in the dark. cFDA was added to a concentration of 50 μM to the cell suspension and the mixture incubated at 30°C for 15 min (or 60 min for cells pre-grown in presence of 8% (vol/vol) ethanol). Immediately after labeling, the cells were spun down, washed once, and resuspended in 50 mM KPi (pH 7.0) to an OD_{600} of 2.0 for fluorimetrical analyses.

Fluorescent labeling with PI. Propidium iodide (PI), a positively charged fluorescent nucleic acid dye was used to stain cells with compromised membranes. Stock solutions of 1.0 mg of PI (Molecular Probes) per ml were prepared in distilled water, stored in the refrigerator, and kept in the dark. Cell suspensions of an OD_{600} of 5 were diluted 1000-fold in 50 mM KPi (pH 7.0) and PI was added to a final concentration of 7.5 μM. Cells were incubated at 30°C for 10 min. For double staining assays, cell suspensions (OD_{600}=0.005) of cF pre-stained cells were used.

Esterase activity. Cell extracts were prepared by disrupting 600-μl portions of cell suspension (OD_{600} = 40) by bead beating (6 times for 30 s with 45-s intervals to cool the samples). The cell debris was removed by centrifugation at 6160 x *g* at 4°C for 2 min. The cFDA hydrolysis activity of cell extracts was

determined by incubation of 100 µl of 1.0 mM cFDA and 250 µl of the cell extract in 50 mM KPi buffer (pH 7.0) in a total volume of 1.0 ml at 30°C. The increase of cF concentration over time was monitored by measuring A_{490} for 20 min, at 5 min intervals. The values were corrected for the chemical hydrolysis of cFDA.

Measurement of cF efflux. cF-loaded cells were washed twice and re-suspended in 50 mM KPi buffer (pH 7.0) to a final OD_{600} of 2.0. At time zero, cell suspensions were placed in a waterbath at 30°C and incubated without and with ethanol (8, 12 and 16% (vol/vol)). Samples (200 µl) were withdrawn at specific time points and immediately centrifuged to remove the cells. To measure the cF labeling capacity, labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed by centrifugation. The fluorescence of the supernatant was measured fluorimetrically (excitation at 490 ± 5 nm and emission at 515 ± 5 nm), with a Perkin-Elmer LS 50B luminescence spectrometer. From the fluorescence of the supernatants and the total labeling capacity, the intracellular concentrations of cF at the sampling points were calculated. For flow cytometry assays a suspension of *O. oeni* labeled cells (OD_{600}=5) was diluted 1000-fold in 50 mM KPi (pH 7.0) to a final cell concentration of approximately 10^6 cells per ml in the absence and presence of ethanol. At time zero, the cells were placed in a waterbath at 30°C. Time series were made by taking 100 µl aliquots which were diluted with 50 mM KPi (pH 7.0) to a final volume of 1 ml and immediately analyzed.

Flow cytometric analysis. The FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used for single-cell light scattering and fluorescence measurements. The samples were illuminated with a 15 mW 488 nm, air-cooled Argon-ion laser, and fluorescence emission was detected at 530 nm for cF and at >670 nm for PI. List mode data from approximately 5000 cells were collected and processed by using the CELLQuest program (version 3.1f; Becton Dickinson). Photomultiplier amplifier gains were set in the logarithmic mode for both light scattering and fluorescence. A combination of forward (FSC) and side scatter (SSC) was used to discriminate bacteria from background. Light scattering and fluorescence were triggered by side angle light scattering, with the threshold limit set to 200 channels, in order to reduce background noise. Data were analyzed with the WinMDI program (version 2.8: Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia [http://jcsmr.anu.edu.au]).

Additional Analytical Methods. Cellular dry weight was determined by filtering 80 ml of the cell suspensions through pre-weighed polyethylene filters, with a porosity of 0.22 µm, and dried at 100°C in an oven until a constant weight was reached. As a control the dry weight of the same volume of phosphate buffer was also determined. Protein was assayed by the method of Lowry et al. (1951).

2.4. Results

Passive proton influx. In order to ensure that passive proton movements were not concealed by proton extrusion through the membrane ATPase, two controls were used. Assays were performed in the presence of either 2-deoxy-D-glucose (a glucose analogue of which the phosphorylation results in depletion of ATP) or DCCD (N,N-dicyclohexylcarbodiimide), an F_0F_1-ATPase inhibitor. The presence of these compounds in the assays did not affect the measurements of H^+ influx, indicating that under the conditions used, there was no significant active proton movement. The passive proton influx was evaluated by the extracellular alcalinization, measured by an acid-pulse titration technique as described in Material and Methods. After the rapid adjustment of the initial pH to 3.5 (pH of wine) or 4.5 (pH of growth) we observed that extracellular pH increased with time in the cell suspensions.

Ethanol induced an increase of the passive proton influx and the rates were higher at low extracellular pH (Fig. 2-1). The exponential enhancement constant of proton influx induced by ethanol was

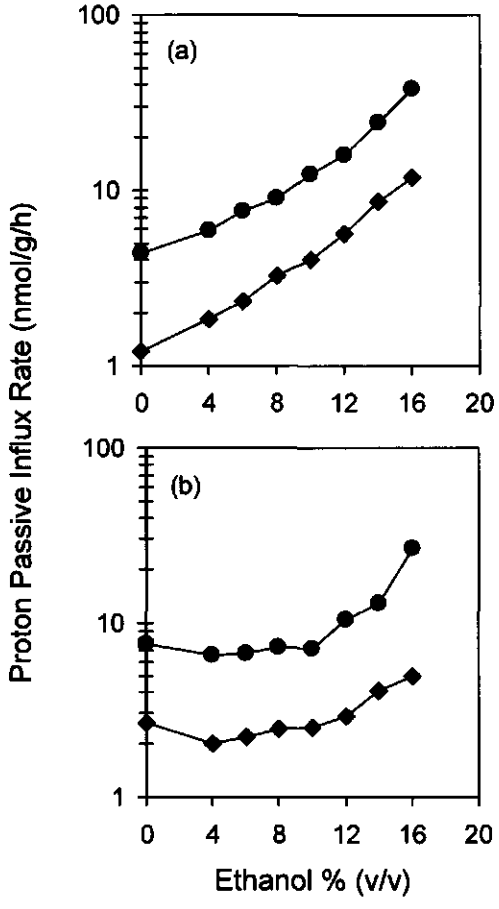

Figure 2-1 Effect of ethanol on the passive proton influx in cell suspensions of *O. oeni* at pH 3.5 (●) and pH 4.5 (◆). Cells grown at pH 4.5 and 30°C in the absence (a) or in the presence of 8%(vol/vol) ethanol (b). The results are the mean of four different assays and were performed at growth temperature.

not significantly affected by the initial extracellular pH and was 0.8 $l.mol^{-1}$ at pH 3.5 and 4.5. Cells grown in the presence of 8% ethanol (vol/vol) (Fig. 2-1b) showed a higher tolerance to ethanol, since for concentrations up to 10 % (vol/vol) lower passive proton influx rates were observed especially at pH 3.5.

Leakage of intracellular compounds. Leakage of intracellular compounds was evaluated immediately after ethanol addition in order to evaluate the instantaneous effect of ethanol shock and also after 5 min of incubation with ethanol. In *Oenococcus oeni* cells exposed for 5 min to increasing concentrations of ethanol the loss of compounds absorbing at 260 nm was stimulated only by concentrations over 8% (vol/vol) of ethanol (Fig. 2-2a). For these concentrations, the extracellular amount of compounds absorbing at 260 nm was clearly correlated with increasing ethanol concentrations. At 16% (vol/vol) of ethanol an immediate loss of compounds absorbing at 260 nm was observed, pointing to an instantaneous disorder of the membrane (Fig. 2-2a). Fig. 2-2b shows the effect of the adaptation on the ethanol-induced membrane disordering, since leakage of compounds was much lower in cells grown in the presence of 8% (vol/vol) ethanol. Comparison of Fig. 2-2a and 2-2b shows that the extracellular

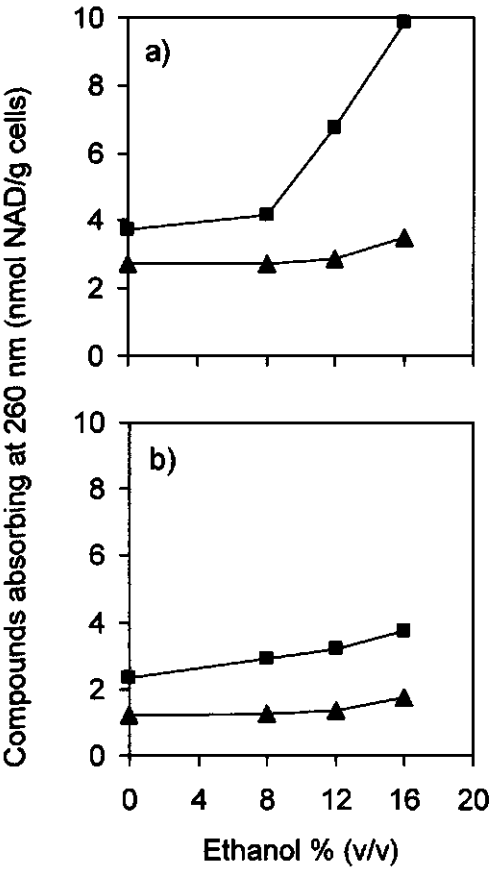

Figure 2-2 Ethanol-induced efflux of compounds absorbing at 260 nm from cells of *O. oeni* grown at 30°C in the absence (a) or in the presence of 8%(vol/vol) of ethanol (b). The efflux values were measured immediately after the ethanol shock (▲) and after 5 minutes of exposure to ethanol (■).

concentrations of compounds absorbing at 260 nm released from adapted cells, after 5 min of incubation with ethanol, are in the same order of the values monitored immediately after ethanol addition in non-adapted cells.

Accumulation of cF. Cells grown with and without ethanol were incubated in the presence of cFDA, and the accumulation of cF was followed in time. In the FCM analysis of *O. oeni* cells the labeled population gave a peak in the green fluorescence histogram, which was resolved from the signal of non-labeled cells. cFDA was efficiently taken up and hydrolyzed by *O. oeni*. Nearly all *O. oeni* cells of a non-adapted cell suspension were labeled with cF within a few minutes of incubation with cFDA while the cells grown in presence of 8% (vol/vol) ethanol took much longer to become fluorescent (Fig. 2-3). Analysis of cF staining of ethanol-grown cells after 15 min resulted in overlapping peaks of labeled and non-labeled cells. Only after 60 min of incubation with cFDA the labeled population gave a peak in the FCM histogram distinct of the non-labeled cells (Fig. 2-3b). This indicates that cFDA uptake is reduced presumably because it is less soluble in the cytoplasmic membrane of ethanol-adapted cells, since both cells grown with or without ethanol had similar esterase activity i.e. 0.067 and 0.066 μmol cF/min/mg of protein, respectively. This implies a change in composition and/or organization of the cytoplasmic membranes of cells grown in presence of ethanol.

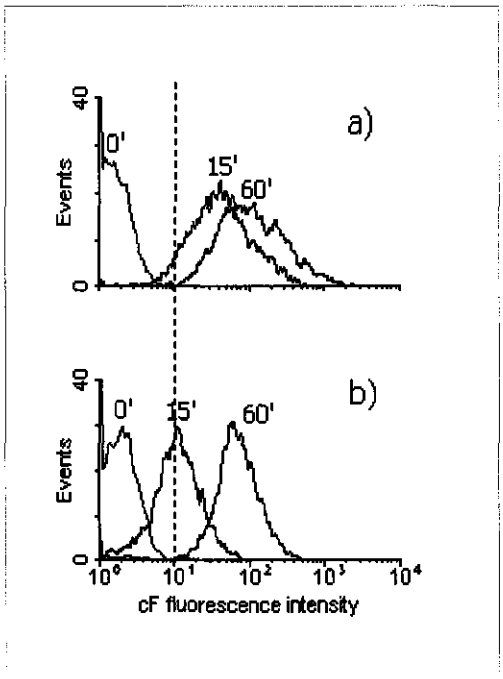

Figure 2-3 Single-parameter histograms of cF uptake in *O. oeni* cells grown without ethanol (a) and in presence of 8% (vol/vol) ethanol (b). Cells were incubated with 50 μM cFDA in KPi buffer (pH 7) at 30°C. Subsequently, the increase of fluorescence after 15, 30 and 60 minutes was measured using FCM.

Ethanol-induced cF leakage. *O. oeni* cells retain cF when not energized, and extrude the probe rapidly by an energy-dependent efflux system (data not shown). De-energized cells of *O. oeni* were loaded with cF and subsequently the cF retention was studied in the presence of increasing concentrations of

ethanol by measuring spectrofluorimetrically the amount of probe released in the supernatant of the cell suspension. In the absence of ethanol a gradual release of cF in the supernatant was observed at a rate of 0.8% per min. In cells incubated with 8% and 12% (vol/vol) of ethanol the rates of cF efflux increased to approximately 1.7% and 2.6% per min, respectively. The permeabilization of cells with 16% of ethanol resulted in a rapid loss of cF, i.e. 50% in 5 min (Fig. 2-4a). *Oenococcus oeni* cells grown in the presence of 8% (vol/vol) of ethanol were able to retain more efficiently cF (Fig. 2-4b), even in the absence of ethanol (rate of 0.4% per min). When these cells were exposed to 8% and 12% (vol/vol) of ethanol the rate values of cF efflux were approximately 1.2% and 1.7% per min, respectively. Notably, challenging pre-adapted cells to 16% (vol/vol) of ethanol, more than doubled the time needed to release 50% of accumulated cF ($t_{1/2}$) compared to that in non-adapted cells (Fig. 2-4b).

When the total cF retained in a suspension of *O. oeni* declines by 50%, this could be because all of the cells released half of cF accumulated or because half of the cells have lost all of the probe. Thus, flow cytometric analysis that enables measurements to be made on individual cells may provide additional

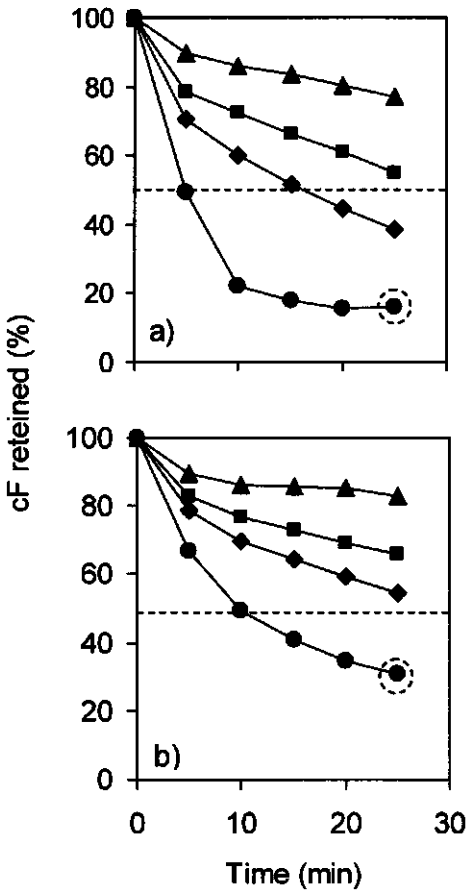

Figure 2-4 Ethanol induced cF efflux in de-energized *O. oeni* cells. Cells were loaded with cF by incubation at 30°C with 50 μM cFDA. The efflux of cF was measured at 30°C in 50 mM potassium buffer (pH 7.0) in non-adapted (a) and in ethanol adapted cells (b). Concentrations of ethanol in the assay: 0 (▲); 8 (■); 12 (◆) and 16 % (vol/vol) (●).

information. The points marked in Fig. 2-4, corresponding to extreme situations, were selected for more detailed analysis by flow cytometry. In the FCM analyses, the *O. oeni* cells were easily detected by their light scatter. In the dotplot of the FSC and the SSC a region was created that comprised the cell population. Interfering particles that also had a SSC above the threshold, but were not in the delineated region were thus disregarded.

In cell suspensions of *O. oeni* grown without ethanol, 98% of the population could be labeled with cF. When these cells were incubated without ethanol they retained efficiently the probe (data not shown). However, when cF-loaded cells were exposed to 16% (vol/vol) ethanol for 25 min the probe was lost by passive diffusion, as determined from the decrease in the fluorescence intensity in the 515 nm fluorescence/side scatter dot plots (Fig. 2-5a). Less than 1% of the cells were considered cF fluorescent. In

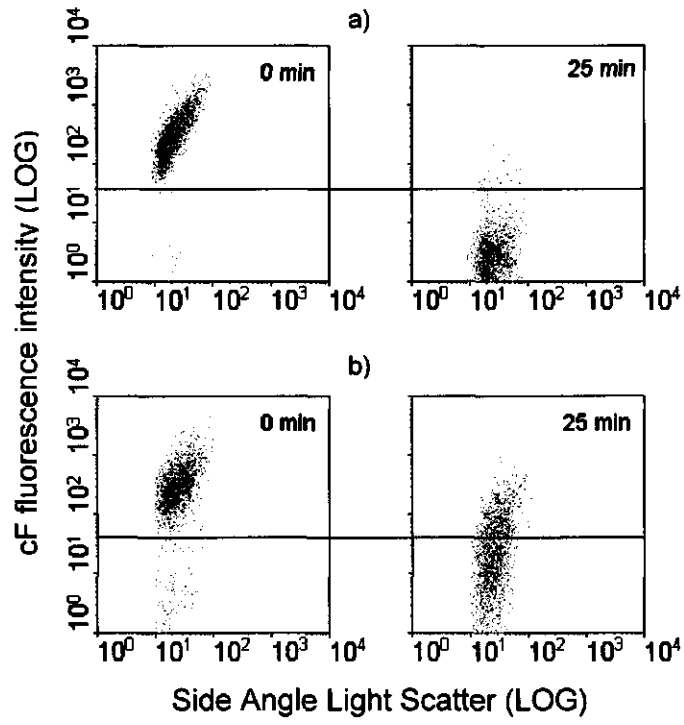

Figure 2-5 Flowcytometric analysis of ethanol-induced cF efflux in *O. oeni* cells. Two-parameter dot plots are shown of cF-loaded cells previously de-energized and incubated with 16% ethanol (vol/vol), at 30°C in KPi buffer (pH 7). Samples were withdrawn immediately after ethanol addition and after 25 min of incubation. cF retention was analyzed by flow cytometry in non-adapted (a) and in ethanol-adapted cells (b).

cells grown in the presence of 8% (vol/vol) ethanol, 95% of the population was cF-labeled, suggesting that adaptation to ethanol resulted in a small additional loss of the labeling capacity of 3% of the population. After incubation of these cells with 16% (vol/vol) ethanol for 25 min, two sub-populations could be distinguished one of which being able to maintain the cF and the other one unable to retain efficiently the probe (27.6%). FCM analysis thus revealed that the population of *O. oeni* is heterogeneous with regard to ethanol resistance based on the cF-retention capacity. These ethanol-stressed cells were also tested for their labeling capacity, and it was found that stressing the cells with 16% ethanol for 25 min resulted in a sub-population that was not labeled with cF in both adapted (16%) and non-adapted cells (25%) (Fig. 2-6). This means that stressing *O. oeni* cells with 16% (vol/vol) ethanol for 25 min resulted in a loss of cF-labeling capacity of 23% and 10% of the population of non-adapted and ethanol-adapted cells, respectively.

PI staining. Flow cytometric analysis of *O. oeni* cells revealed two sub-populations corresponding to damaged cells, i.e. cells that were PI stained, and to cells with an intact cytoplasmic membrane, which were not stained. By growing cells in the presence of 8% (vol/vol) ethanol, 5% of the population lost their membrane integrity, compared with 3% of the non-adapted cells. These results are confirmed by the results of cF labeling capacity (Fig. 2-6) and signify that the adaptation of *O. oeni* cells to ethanol is associated with increased membrane damage of a small part of the population. Furthermore, the percentage of cells PI stained remained constant during the treatments performed for flow cytometric analysis (data not shown).

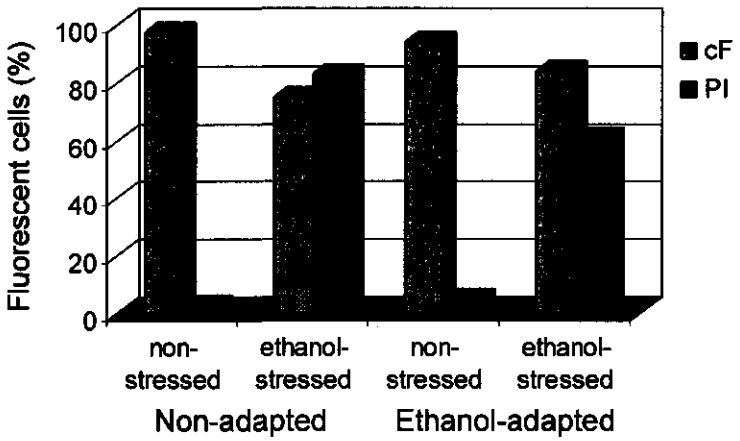

Figure 2-6 Effect of ethanol on fluorescence labeling of *O. oeni* with fluorescent probes. Cells were stressed with 16% (vol/vol) for 25 min, the cells were washed with KPi buffer (pH 7) and labeled in the same buffer at 30°C. PI exclusion and cF retention were analyzed by flow cytometry in non-adapted and 8% ethanol (vol/vol)-adapted cells.

Ethanol-stressed *O. oeni* cells showed a strong increase in permeability to PI (Fig. 2-6). The PI-stained sub-population after exposure to 16% (vol/vol) ethanol for 25 min was 83% in non-adapted cells and 61% in ethanol-adapted cells. The positive effect of adaptation is then confirmed by the fact that in ethanol-adapted cells only 56% of the population lost the capacity to exclude PI, compared to 80% of the population of non-adapted cells.

Double staining with cFDA and PI. Although we observed an increase of PI staining in these ethanol-stressed cells, which point to a complete damage of that sub-population, the fraction of cF-labeled cells remained high (Fig. 2-6). These results suggest that ethanol-stress induces a transient influx of PI into *O. oeni* cells. Therefore, both probes were used in combination to gain additional information about the physiological state of these ethanol-stressed cells. Staining the cells with a mixture of the two fluorescent dyes cF and PI revealed three main sub-populations corresponding to intact, cF stained cells (A); permeable, cF-PI double-stained cells (B); and damaged, PI-stained cells (C) (Fig. 2-7). It is evident that only a minority of the cells (12%) has maintained their membrane integrity (A), i.e. stained only with cF (Fig.

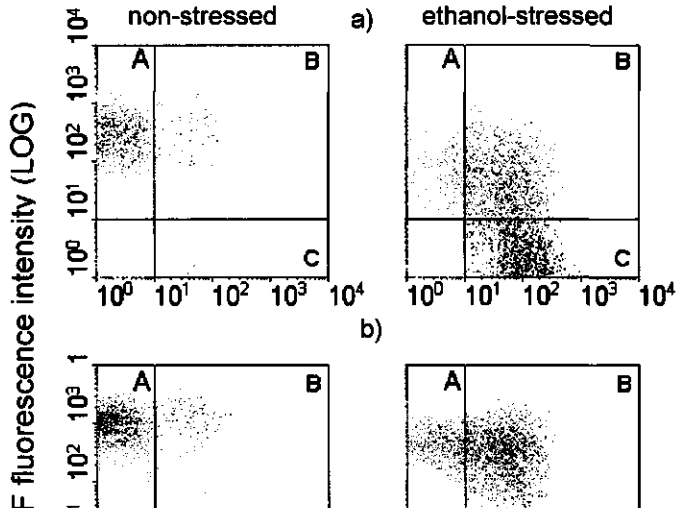

Figure 2-7 FCM analysis of PI-cF double stained *O. oeni* cells stressed with 16% ethanol (vol/vol) for 25 min. Three main sub-populations of cells were identified, in both non-adapted (a) and ethanol-adapted cells (b), which correspond to cF stained, intact cells (A); cF-PI double stained cells in intermediate state of permeability (B); and PI-stained, damaged cells (C).

2-7a). Approximately 35% of the PI-stained cells also accumulated cF (B), indicating a progressive change in the physiological status of *O. oeni*, as has become evident by this sub-population with intermediate membrane permeability. In cells grown in the presence of 8% (vol/vol) ethanol and challenged for 25 min to 16% (vol/vol) ethanol, the sub-population that stains with both cF and PI (B) represents 47% of the total population (Fig. 2-7b). However, the PI-stained sub-population is significantly smaller i.e. 14%, compared with cells grown without ethanol, while the sub-population corresponding to intact cells is much larger (39%).

2.5. Discussion

This report clearly shows that ethanol acts as a disordering agent of the *O. oeni* cytoplasmic membrane, leading to leakage of intracellular material absorbing at 260 nm and promoting the passive influx of protons. The inability of ethanol-stressed cells to retain cF efficiently also indicates that ethanol promotes an increase of the permeability of the *O. oeni* cytoplasmic membrane. Uptake of PI by *O. oeni* cells after exposure to ethanol further points to the ethanol-induced membrane damage. The observed effects suggest that failure of MLF after direct inoculation of *O. oeni* in wine, may be explained by the deleterious effects of ethanol in combination with low pH of the wine.

First-order kinetics is commonly used to estimate permeability. However, the kinetics of passive efflux of intracellular compounds was complex and the rate constant couldn't be easily derived (data not shown). Therefore, we used the leakage of the fluorescent probe cF to monitor the permeability of ethanol-stressed cells. Ethanol-induced membrane damage assessed by fluorimetric assays did not reflect the effect of ethanol on permeability of cytoplasmic membrane as monitored by leakage of compounds absorbing at 260nm. The leakage of these intracellular compounds was only stimulated at ethanol concentrations above 8% (vol/vol). Typical compounds absorbing at 260 nm include NAD^+, NADH and AMP (25, 30). These molecules have significantly higher molecular mass than cF, i.e. that of NAD^+ is 663 g per mol and cF 376 g per mol. Consequently, higher membrane disorganization is probably necessary to occur in order to promote the leakage of intracellular compounds absorbing at 260 nm. This hypothesis is supported by the fact that cF leakage followed the global trend of ethanol concentration dependence that paralleled the increase of passive proton influx rates at increasing ethanol concentrations.

NAD^+ is an important cofactor of the malolactic enzyme and it has been reported that the delicate balance between NAD^+/NADH affects the activity of the enzyme (28). Conceivably unspecific leakage of intracellular material will disturb significantly this balance, and consequently it is expected that cell metabolism will be negatively affected. Moreover, it is obvious that ethanol-induced proton influx will affect physiological processes in *O. oeni* that are dependent on a pH gradient, like ATP synthesis, the transport of L-leucine (39) and L-malate uptake (40, 32). Notably, Capucho and San Romão (1994) observed that at pH 3.0, ethanol inhibits the malolactic activity at concentrations above 12% (vol/vol) ethanol, while at pH 5.0 no effect was detected at concentrations up to 20% (vol/vol) ethanol. This supports the idea that ethanol

inhibits MLF by enhancing passive proton influx at low extracellular pH, decreasing the intracellular pH to values not suitable for malolactic enzyme activity for which the optimum pH is 5.5 (3). MLF failure after direct inoculation of *O. oeni* in wine can be caused by cytoplasmic membrane disorder induced by ethanol in combination with low pH of the wine.

We also analyzed the effect of pre-adaptation to ethanol followed by exposure to high concentrations of this compound. Although permeabilization was induced by ethanol, cells grown in the presence of this compound were more resistant. Such cells showed lower passive proton influx rates at ethanol concentrations higher than 10 % (vol/vol), especially at pH 3.5. *Oenococcus oeni* cells grown in the presence of 8% (vol/vol) ethanol also presented higher intracellular retention of compounds absorbing at 260 nm.

It is now well established that the survival of bacteria in a variety of potentially lethal conditions can be enhanced by pre-exposure to sub-lethal stress conditions of the same kind (12, 18, 11). In this work we demonstrate that this acquisition of tolerance is not a phenomena observed at the population level but at the level of the single cells. Flowcytometric analysis of ethanol-adapted cells showed that the population is heterogeneous with respect to adaptation to ethanol. Thus not all the cells developed a mechanism that increased partially the efficiency of their cytoplasmic membrane as a barrier, i.e. one sub-population became completely efficient in retaining cF, while the other was completely ethanol-sensitive.

Our labeling experiments showed that ethanol-adapted cells of *O. oeni* take much longer to become cF fluorescent than non-adapted cells. This phenomenon can be explained by the difference in membrane permeability characteristics affecting the diffusion of cFDA. This suggests that cFDA is less soluble in the cytoplasmic membrane of cells grown in presence of 8% (vol/vol) of ethanol, since both cells presented the same esterase activity. This result confirms the hypothesis that mechanisms involved in ethanol adaptation are associated with modification of the membrane composition, aiming to maintain the optimal activity of several biological processes (11). In *O. oeni* different stresses dramatically induce the expression of an 18-kDa small heat shock protein (Lo18) that was found to be peripherally associated with the membrane (15). The authors observed the synthesis of Lo18 after incubation of cells in the presence of 12% ethanol, however no induction was found at 10% (vol/vol) ethanol (14). This strongly supports the idea that the ability of this bacterium to adapt during growth at 8% (vol/vol) ethanol is related with the capacity to regulate the membrane composition. Actually, it has been firmly established that fatty acid composition of microbial cells is often modified in response to environmental changes (38, 20, 19, 21). Garbay et al. (1995) observed that the fatty acid composition of *O. oeni* membrane, varied when cells had been grown in the presence of wine. These conditions induced a two-fold increase in the ratio unsaturated/saturated fatty acids.

The double staining of ethanol-stressed cells with cFDA and PI revealed interesting population heterogeneity. It is generally assumed that bacterial permeability to nucleic acid binding dyes such as PI is associated with the presence of irreparable breaches in the membrane (37), making it at the same time

impossible for the cells to retain cF (36, 8). However, in this work we found large sub-populations of cF and PI double-stained ethanol-stressed cells, showing that such assumptions are too simplistic and indicate that the mechanisms of action of ethanol may be more complex than commonly perceived. A PI-cF double staining has also been observed in bile salt stressed bifidobacteria cells (Ben-amor et al, unpublished). These results support the idea that dye exclusion assays should be designed to reflect the complexity of the process being investigated rather than to estimate cell death (43). Especially, if stress factors or treatments that target the cell membrane are involved.

The size of the sub-population of cells in an intermediate state of membrane damage (double stained cells) was similar in both adapted and non-adapted cells. However, the positive effect of ethanol adaptation is demonstrated by the fact that the sub-population of *O. oeni* cells that have maintained their membrane integrity, i.e. cells only stained with cF, was three times larger in cells grown in the presence of ethanol. Moreover, the sub-population corresponding to damaged cells, i.e. stained only with PI, was almost four times smaller in these ethanol-adapted cells. Our results show that simultaneous assessment of changes in two physiological characteristics, esterase activity and membrane composition, by multiparametric flow cytometry allows distinction between different levels of ethanol-damage in ethanol-adapted populations. This information is of major importance in the study of MLF in wine, in order to assign bulk activities measured by classical methods to the very active cells of the starter culture that are effectively responsible for the observations. Sorting of these sub-populations and further physiological analysis represents a powerful strategy for the understanding of the mechanisms involved in ethanol stress response and tolerance.

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3. MEMBRANE FLUIDITY ADJUSTMENTS IN ETHANOL-STRESSED *OENOCOCCUS OENI* CELLS

3.1. Abstract

The effect of ethanol on the cytoplasmic membrane of *Oenococcus oeni* cells and the role of membrane changes in the acquired tolerance to ethanol were investigated. Membrane tolerance to ethanol was defined as the resistance to ethanol-induced leakage of preloaded carboxyfluorescein (cF) from cells. To probe fluidity of the cytoplasmic membrane, intact cells were labeled with doxyl-stearic acids and analyzed by electron spin resonance spectroscopy. Although the effect of ethanol was noticeable across the width of the membrane we focused on fluidity changes at the lipid-water interface. Fluidity increased with increasing concentrations of ethanol. Cells responded to growth in the presence of 8% (vol/vol) of ethanol by decreasing fluidity. Upon exposure to a range of ethanol concentrations, these adapted cells had reduced fluidity and cF-leakage as compared with cells grown in the absence of ethanol. Analysis of the membrane composition revealed an increase in the degree of fatty acid unsaturation and a decrease in the total amount of lipids in the cells grown in the presence of 8% (vol/vol) of ethanol. Pre-exposure for 2h to 12% (vol/vol) of ethanol also reduced membrane fluidity and cF-leakage. This short-term adaptation was not prevented in the presence of chloramphenicol, suggesting that *de novo* protein synthesis was not involved. We found a strong correlation between fluidity and cF-leakage for all treatments and alcohol concentrations tested. We propose that the protective effect of growth in the presence of ethanol is, to a large extent, based on modification of the physicochemical state of the membrane, i.e. cells adjust their membrane permeability by decreasing fluidity at the lipid-water interface.

Da Silveira, M. G., E. A. Golovina, F. A. Hoekstra, F. M. Rombouts and T. Abee. 2003. Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. Appl. Environ. Microbiol. Accepted.

3.2. Introduction

Malolactic bacteria are lactic acid bacteria that are able to carry out malolactic fermentation (MLF). The control of their activity is an important aspect of the technology of commercial wine production. MLF consists of the decarboxylation of L-malic acid to L-lactic acid, which decreases total acidity and improves the stability and quality of wine. *Oenococcus oeni* is recognized as the principal microorganism responsible for MLF under stress conditions, such as those prevailing in wine. However, inoculation of *O. oeni* starter cultures directly into wine leads to significant cell mortality and, consequently, failure of MLF. The reactivation and adaptation of starter cultures to wine conditions prior to inoculation into wine considerably enhance survival of the bacteria (For a review in MLF see [29]). Ethanol tolerance appears to be a crucial parameter for the activity of *O. oeni* cells in wine.

The role of ethanol as an agent affecting the physicochemical state and biological functions of various cell membranes has been extensively studied. Ethanol toxicity is now generally attributed to the interaction of ethanol with membranes at the aqueous interface, resulting in a perturbed membrane structure and function (47). In an extensive review about the biological effects of ethanol, Jones (18) argues that membrane-located effects of ethanol are the result of the dielectric disruption of the aqueous phase, of competition with water for membrane polar sites, and of selective location within the polar region of membrane surfaces or proteins. However, the exact range of biochemical and physiological processes affected by ethanol is, for the most, undefined.

The importance of the membrane lipid composition with respect to ethanol tolerance has been extensively studied in yeast (45, for a review see [35]) and bacteria (12, 22). Ethanol tolerance has been strongly correlated with adaptive changes in plasma membrane composition, with many studies in yeast suggesting a role for acyl chain unsaturation (32, 44, 45).

Ethanol tolerance has been associated with high plasma membrane fluidity both in yeast (1, 39) and bacteria (3, 10). The fluidization response can be interpreted on the basis of the hypothesis of "homeoviscous adaptation" (40) as a counteraction to the physico-chemical effect of ethanol on membranes (22). This model, whilst being widely reported, is apparently not universally applicable to all organisms. Exceptions have been reported in *B. subtilis* (36) and *E. coli* cells (12), in which plasma membranes isolated from cells grown in the presence of ethanol were more rigid than those from the control cells.

While membrane lipid composition has been considered important for cellular stress tolerance, also other factors have received extensive consideration. One widely studied aspect of the ethanol-stress response is the induction of heat shock proteins (hsps) (23, 30). However, the relative contribution of hsps to ethanol tolerance has been questioned (11, 41). Although hsps and membrane composition are both

likely to be of importance in ethanol tolerance, the relative contribution of each and the mechanisms of action remain unresolved.

The degree to which the effects of ethanol on membrane composition and fluidity share common features has not previously been explored in *O. oeni*. In addition, no direct measurements of the effect of growth in the presence of ethanol on the mobility of membrane components have been established. In this report we investigated the contribution of membrane fluidity changes to ethanol-stress tolerance and the relationship of those changes with the changes in fatty acid composition of *O. oeni* membranes. Ethanol-stress tolerance was examined by following the leakage of preloaded carboxyfluorescein (cF) from the cells. The effect of ethanol challenge on the organization and dynamics of the plasma membrane in intact cells of *O. oeni* was assessed by *in vivo* spin label technique. Three nitroxide spin labels were used to obtain motional anisotropies of the nitroxide moiety at different depths of the plasma membrane. The molecular order parameter S derived from electron spin resonance (ESR) spectra provided a measure of membrane structural order. This parameter was studied in relation to the concentration of ethanol in non-adapted cells and in ethanol-adapted cells grown in presence of 8% ethanol or after a short exposure to 12% ethanol. This study was undertaken to test the hypothesis that ethanol may be toxic to *O. oeni* because of its effects on the plasma membrane and that adaptation can partially or completely reverse these membrane effects via changes in membrane composition and/or organization. Adaptation was assessed as the reduction in ethanol-induced cF leakage. The relationship between membrane order and acyl-chain composition in the tolerance of *O. oeni* cells is discussed.

3.3. Materials and Methods

Strain, medium and growth conditions. *Oenococcus oeni* GM (Microlife Technics, Sarasota, Fla.) was cultured at 30°C in FT80 medium (7) at pH 4.5 without the addition of Tween 80, but containing 10 g of DL-malic acid per liter. Glucose and fructose were autoclaved separately and added to the medium just before inoculation at a final concentration of 2 and 8 g per liter, respectively. Stock cultures (kept frozen at -80°C) were grown until early stationary phase (48 h), diluted 100-fold in fresh medium, incubated for 24 h, and then used to obtain 1% inoculated cultures.

Cell adaptation. For adaptation to ethanol during growth, *O. oeni* cells were cultured at 30°C in 500 ml of FT80 medium, pH 4.5, with 8% (vol/vol) of ethanol and recovered after 48 h (late exponential). For short-term adaptation, *O. oeni* was cultured in 500 ml of the same medium at 30°C. Exponential phase cells (24 h) were harvested by centrifugation, suspended in the same medium containing 12% (vol/vol) of ethanol and incubated for two hours at 30°C. The same procedure was repeated in the presence of chloramphenicol (80 μg/ml) in order to inhibit *de novo* protein synthesis as observed by Jobin et al in *O. oeni* (24).

ESR spectroscopy. Membrane fluidity of intact *O. oeni* cells was studied by ESR spin probe technique. Spin-labeled stearic acids were used to probe membrane fluidity. In this molecule the nitroxide

doxyl group (stable radical) is attached in a rigid, stereo-specific manner to stearic acid so that the motion of the nitroxide group directly reflects the motion of the labeled part of stearic acid. The ESR spectral shape of spin-labeled stearic acids depends on the motion and angular orientation of the nitroxide group with respect to the membrane lipid-water interface (31). Depending on the position of the doxyl group along the carbon chain (at the 5, 12, or 16th C-atom), it is possible to probe the motional freedom in membranes at the lipid-water interface, in the middle of the monolayer, and in the core of the bilayer, respectively.

5-doxyl stearic acid (5-DS) is commonly used to probe the membrane lipid-water interface. A typical ESR spectrum of 5-DS-labeled *O. oeni* cells is presented in Fig. 3-1. The anisotropic character of the spectral shape results from the restricted angular freedom of the radical group of 5-DS in the plasma membrane. The spectral parameters $A_{||}$ and $A_{\perp}$ indicate the outer and inner hyperfine splittings in experimental spectra (as shown in Fig. 3-1). The membrane order parameter S relates to membrane fluidity and can be calculated as the ratio between the observed hyperfine anisotropy ($A_{||} - A_{\perp}$) to the maximum theoretically obtainable value of 25 Gauss, which corresponds to the completely rigid orientation of 5-DS (28). Thus, the order parameter can be calculated as follows:

$$S = (A_{||} - A_{\perp})/25 \qquad (1)$$

From this formula it is clear that S=1 for completely rigid order and S=0 for completely isotropic motion. This means that in the case of completely rigid orientation of 5-DS $A_{||}$ is maximal and $A_{\perp}$ is minimal, and in the case of completely isotropic motion the outer and inner splittings are equal. With membrane fluidization $A_{||}$ decreases and $A_{\perp}$ increases, so that the order parameter decreases.

ESR spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker, Rheinstetten, Germany, model 300E). Microwave power was 5 mW, the modulation amplitude was 3 Gauss, and the scan range was 100 Gauss. All spin probes were from Sigma, St Louis, MO, USA and stored as a 0.1M stock solution in ethanol at –20°C.

Cells labelling. For labeling, 1-mM spin probe solution was freshly prepared from the stock solution by dilution in water. Cells (250 ml) were recovered and washed three times with 5 mM EDTA plus 0.15 M KCl in P2 buffer (KPi 30 mM, 0.34 M CH_3COONH_4; pH 7.0) to chelate manganese ions. The cells were resuspended in the same buffer and 25 µl of the cell suspension was introduced in a 2-mm (inner diameter) capillary tube, centrifuged, resuspended in 20 µl of 1-mM spin probe solution and incubated for 2 min. The cells were subsequently centrifuged, and the supernatant was removed completely. To avoid reduction of the nitroxide spin probe, the cells were washed with ferricyanide (120 mM), and after centrifugation and removal of the supernatant, the pellet was ready for ESR spectra recording.

Stress conditions. After the ESR measurements of the control cells (non-stressed), the pellet in the capillary was resuspended into 20 µl of 100 mM ferricyanide. From this, a 25 µl aliquot was mixed with 25 µl of a solution with twice the desired concentration of ethanol in 100 mM ferricyanide. This cell suspension was then divided into two capillaries, one was centrifuged immediately, and the other after 15

min. ESR spectra were recorded in both pellets. ESR measurements were repeated in the same cells after washing out the ethanol by resuspending the pellets in 20 µl of 100 mM ferricyanide for 10 min.

Loading cells with carboxyfluorescein (cF). Cells were harvested at the end of the exponential growth phase (OD_{600}≈0.4) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 20. Cells were de-energized with 2-deoxyglucose (at a final concentration 2 mM) by incubation at room temperature for 30 min to avoid cF extrusion from the cells

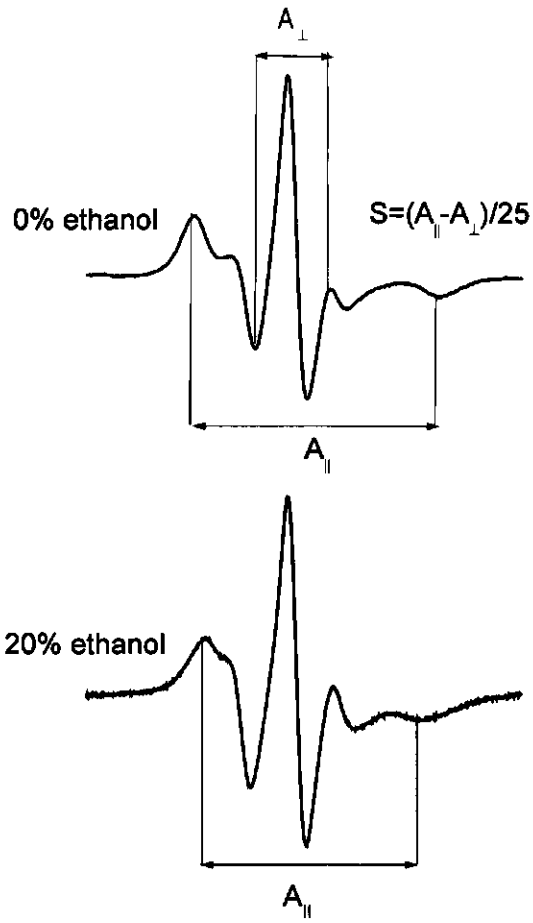

Figure 3-1 EPR spectra of 5-DS in *O. oeni* cells in the absence or presence of 20% (vol/vol) of ethanol. $A_{||}$ and $A_{\perp}$ represent the outer and inner hyperfine splittings, respectively. The order parameter S is calculated as indicated in the figure.

by energy-dependent pumps (5, 6) and consequently to assure that cF-leakage is a consequence of ethanol-induced membrane damage. The cells were washed and resuspended in 50 mM KPi buffer (pH 7.0) to an OD_{600} of 20. A stock solution of 2.3 mg of 5(6)-carboxyfluorescein diacetate (cFDA) (Molecular probes, Eugene, Oregon) per ml was prepared in acetone and stored at -20°C in the dark. cFDA was added to the cell suspension to a final concentration of 50 µM and kept at 30°C for 15 min or 60 min in the case of cells pre-grown in presence of 8% (vol/vol) of ethanol. Immediately after labeling, the cells were spun down, washed once, and resuspended in 50 mM KP_i (pH 7.0) to an OD_{600} of 2.0 for fluorimetrical analyses. Inside cells the uncharged, esterified, pre-fluorochrome cFDA is converted by cytoplasmatic esterases into fluorescent cF that is negatively charged at physiological pH and, consequently, will accumulate inside cells with an intact cytoplasmic membrane (19).

Measurement of cF efflux. cF-loaded cells were washed twice and resuspended in 50 mM KPi buffer (pH 7.0) to a final OD_{600} of 2.0. At time zero cell suspensions were placed in a water bath at 30°C and incubated without and with ethanol [0, 8, 12 and 16% (vol/vol)]. Samples (200 µl) were withdrawn at intervals and immediately centrifuged to remove the cells. To measure the cF labeling capacity, labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed by centrifugation. The fluorescence of the supernatant was measured with a Perkin-Elmer LS 50B luminescence spectrometer (excitation wavelength at 490 ± 5 nm and emission wavelength at 515 ± 5 nm). From the fluorescence of the supernatants and the total labeling capacity the intracellular concentrations of cF at the sampling time points were calculated

Fatty acid analysis. Total lipids were extracted with chloroform-methanol-water from 30 to 40 mg (dry weight) of cells according to Bligh and Dyer (6) and methyl esterified by a 15 min incubation at 95°C in boron trifluoride-methanol (33). The fatty acid methylesters were extracted with hexane, separated on a CP-Sil-88 fused silica capillary column (Chrompack, 50 m x 0.25 mm x 0.20 µm film thickness) and analyzed by GC-MS (Hewlett Packard, 5970B-series GC-MS). Electron impact spectra were obtained at 70 eV electron energy. The following operating conditions were used: injection temperature: 250°C; oven temperature: initially 50°C, rising to 275°C at 6°C/min, maintained at this temperature for 10 min. The fatty acids were identified with the aid of fatty acid methylester standards (Sigma, St Louis, MO, USA), and the identity was confirmed using the NIST Mass Spectral Library. Replicate determinations indicated that the relative error (standards deviation of the mean x 100%) of the values was less than 8%. The average results of three independent experiments are presented. In semi-quantitative analysis, the percentage of each fatty acid was calculated by the ratio peak area/sum of total identified peak areas x 100. In quantitative analysis, peak areas were related to that of the internal standard (C_{22}) and then converted to µg using the area of the nearest standard peak for the calculation.

3.4. Results

Ethanol-induced cF leakage. The ethanol-induced leakage of the fluorescent probe cF from *O. oeni* cells into the supernatant was studied to determine the cellular resistance to the disruptive effect of ethanol. The increased cF-leakage rate values at high ethanol concentrations indicated that cell membranes were sensitive to ethanol in all the conditions shown (Fig. 3-2). Exposure of nonadapted control cells to 16% ethanol resulted in a rapid loss of cF, suggesting an immediate disorganization of the plasma membrane. However, cells grown in the presence of 8% (vol/vol) of ethanol were able to retain cF more efficiently, for all the concentrations of ethanol tested, and even in the absence of ethanol. Leakage rates in these adapted cells were less than 50% of those in nonadapted control cells. *O. oeni* cells pre-incubated with 12% (vol/vol) of ethanol for 2 hours were less leaky for cF during the challenge with increasing ethanol concentrations than the non-adapted cells. The presence of chloramphenicol (CAP) during this 2-h incubation in 12% (vol/vol) of ethanol did not significantly change the cF-efflux kinetics

Ethanol stress and adaptation at the membrane lipid/water interface. Because the interaction of ethanol with the membrane is thought to occur at the membrane lipid/water interface (2, 14, 21), we studied the effects of ethanol using the lipid/water interface membrane probe, 5-DS. Figure 3-3a shows the effect of ethanol addition to intact *O. oeni* cells on the order parameter S, as calculated from ESR spectra of 5-DS (Fig. 3-1). The S-value in non-adapted cells was 0.71 and decreased to 0.58 directly upon the

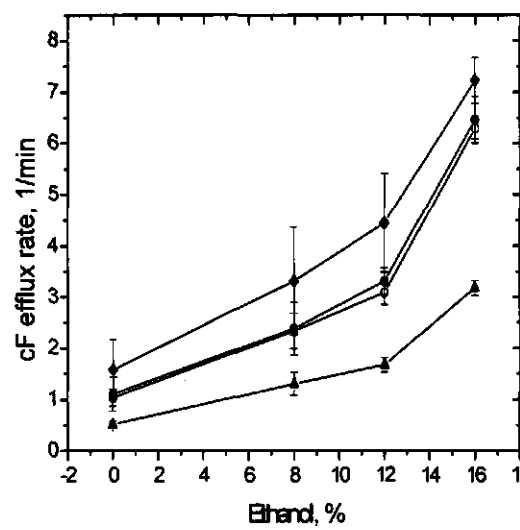

Figure 3-2 Effect of ethanol on the rate of cF efflux from de-energized O. oeni. The cells were loaded with cF by incubation at 30°C with 50 mM cFDA. The efflux of cF was measured by spectrofluorimetry at 30°C in 50 mM potassium buffer (pH 7.0) in cells grown without ethanol (♦), pre-exposed to 12% (vol/vol) of ethanol for 2 hours in the absence (○) and in presence of CAP (●), and grown in presence of 8% (vol/vol) of ethanol (▲).

addition of 20% (vol/vol) of ethanol. Such behavior is indicative of significant, instantaneous disordering of the membrane lipid/water interface. The data on membrane order closely parallel those assessing ethanol effects on cF-leakage. Interestingly, the value of 12% (vol/vol) of ethanol appears to be the point for the

onset of drastic increases in both membrane fluidity and permeability. The shape of the spectra did not change when spectra were rerecorded within 15 min of the initial recording (data not shown), indicating that ethanol causes an immediate disordering effect. Spectra of 5-DS were recorded also from cells grown in the presence of 8% (vol/vol) of ethanol. The value of S obtained from these adapted cells was approx. 0.03 units higher than that from non-adapted cells, indicating higher order at the membrane lipid-water interface (Fig 3-3a). Stressing these ethanol-adapted cells with increasing concentrations of ethanol showed that the S values were always significantly higher than those in the non-adapted cells. The difference in S was particularly evident at the highest concentration of ethanol tested (20%). These results indicate that cells grown in the presence of ethanol are more resistant to ethanol-induced disordering.

In *O. oeni* cells, incubation with 12% (vol/vol) of ethanol induces enhancement of the synthesis of a specific stress protein Lo18, and a significant amount of this protein has been found to be associated with the membrane (23). Based on this, it seemed reasonable to assume that membrane resistance to ethanol disordering is associated with *de novo* protein synthesis, a possibility that we tested by a short adaptation of cells (without growth) to ethanol both in the presence and absence of CAP. Values of S calculated from

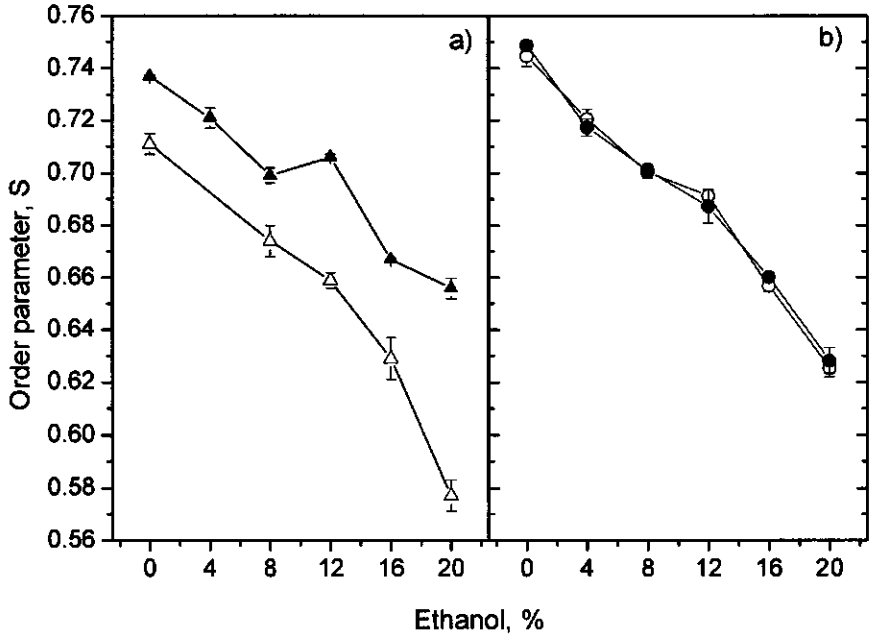

Figure 3-3 The effect of ethanol on the molecular order parameter S calculated from ESR spectra of 5-DS-labeled intact *O. oeni* cells. The measurements were made in (a) non-adapted cells (△); cells grown in the presence of 8% (vol/vol) of ethanol (▲) and (b) cells pre-exposed to 12% (vol/vol) of ethanol for 2 h in the presence (○); or in the absence of CAP (●).

5-DS spectra in cells pre-exposed to 12% (vol/vol) of ethanol for 2 hours were higher than those in control cells (Fig. 3b), i.e. the lipid-water interface region of the membranes in cells pre-exposed to ethanol was more rigid than that in the non-adapted cells. The ethanol concentration-dependent decrease of S in these ethanol-exposed cells followed the same trend as that in the control cells, although the spin label was always less mobile. When cells were pre-exposed to 12% (vol/vol) ethanol in the presence of the protein synthesis inhibitor CAP, the results obtained were similar to those in the absence of CAP at all concentrations of ethanol tested. These results suggest that *de novo* protein synthesis does not play a significant role in membrane adaptation to ethanol-induced disorder under non-growing conditions.

After stressing control and ethanol-adapted cells with increasing concentrations of ethanol, cells were resuspended in a solution of ferricyanide in order to wash out the ethanol. The order parameter was only partially recovered (Fig. 3-4), with the extent of recovery being more evident for the higher concentrations tested. In cells grown in the presence of ethanol the extent of recovery was less pronounced than in non-adapted cells for all the ethanol concentrations tested, with no recovery for cells

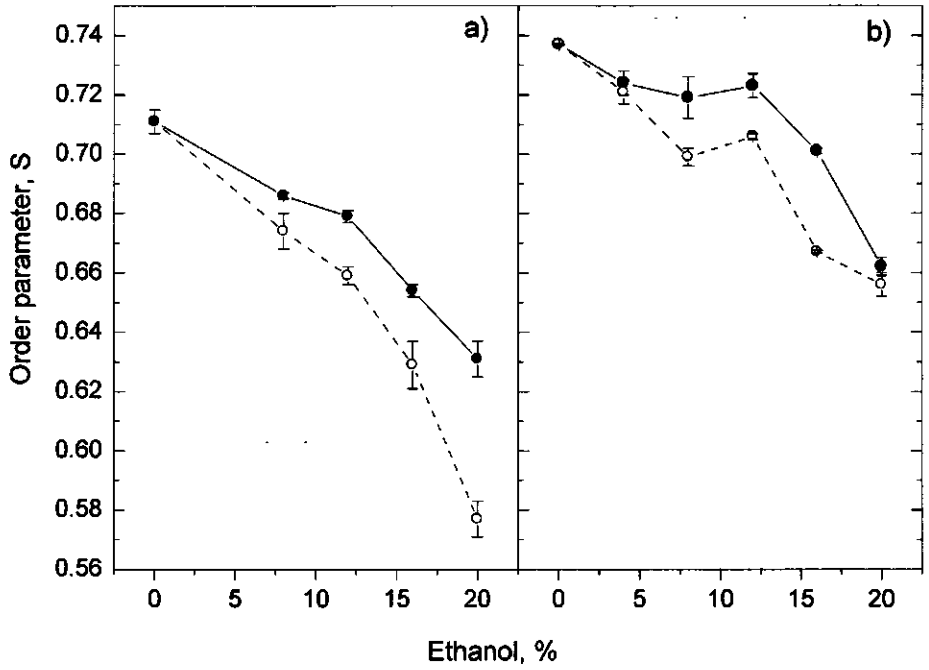

Figure 3-4 The effect of ethanol on the molecular order parameter S calculated from ESR spectra of 5-DS-labeled intact *O. oeni* cells. The measurements were made in non-adapted cells (a) and cells grown in the presence of 8% (vol/vol) of ethanol (b), in washed cells (●) and non-washed cells as the control (○).

stressed with 20% (vol/vol) of ethanol (Fig. 3-4b). A possible explanation for these observations is that pre-exposure to ethanol decreases the partitioning of ethanol into *O. oeni* membranes as it was observed in a variety of other biological membranes (27, 37). Moreover, the observation that the partition coefficient of ethanol correlates inversely with the lipid order (34) is in line with the proposed explanation.

Ethanol stress at the membrane interior. To study the influence of ethanol deeper in the bilayer, we labeled *O. oeni* cells with 12-DS and 16-DS. These compounds probe membrane mobility at the level of the 12th and the 16th carbon atom of the acyl chains, respectively. Figure 3-5 shows a 12-DS spectrum (A, top) and a 16-DS spectrum (B, top) from cells in the absence of ethanol. The shapes of these spectra were different from that of 5-DS (Fig. 3-1) and are indicative of an increasing motional freedom towards the lipid hydrocarbon core (26). The presence of 20% (vol/vol) of ethanol causes narrowing of the lines of both the 12-DS and the 16-DS spectra (bottom spectra in Fig. 3-5A and B), which can be interpreted to mean that membrane fluidity has increased (18). Thus, ethanol has a fluidizing effect on *O. oeni* membranes not only at the membrane lipid/water interface, but also deeper in the bilayer. Adaptation to ethanol does not cause notable changes in spectral shape of 12-DS and 16-DS (spectra not shown), thus suggesting that the effect of adaptation mainly relates to the membrane lipid/water interface.

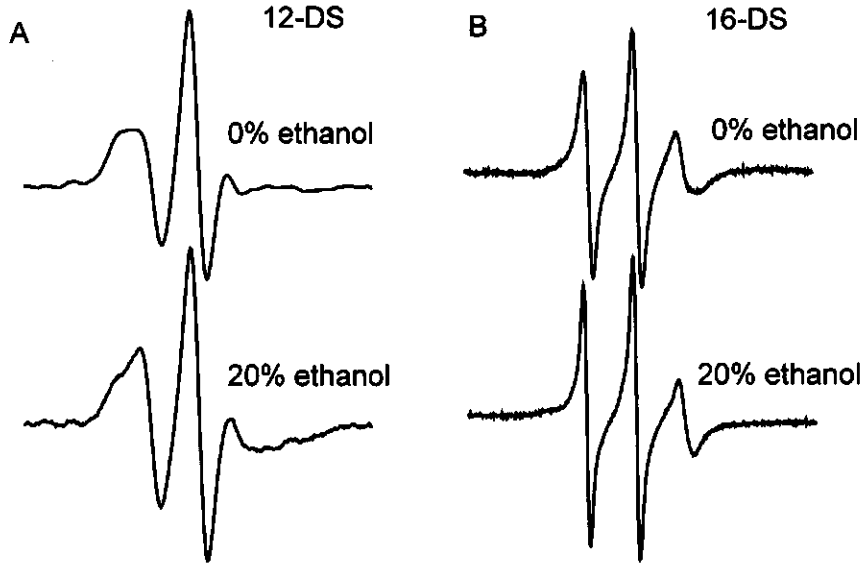

Figure 3-5 EPR spectra of doxyl stearate spin probes 12-DS (A) and 16-DS (B) in *O. oeni* cells in the absence or presence of 20% ethanol.

Modification of fatty acyl composition of the phospholipid fraction by different treatments. Membrane lipid fatty acid (FA) composition has been claimed to play a major role in determining membrane fluidity, especially in bacteria that typically lack sterols in their membranes (38). We determined the fatty acyl composition of the phospholipid fraction extracted from *O. oeni* cells grown under normal conditions (control), pre-exposed to 12% ethanol for 2 h in the presence or absence of CAP, or grown in the presence of 8% (vol/vol) of ethanol. Palmitic acid (C16:0) was the major component of the FA profile followed by myristic acid (C14:0) (data not shown). The FA profile found was essentially equal to the results for *O. oeni* reported elsewhere (13, 16, 42), except that we could not detect lactobacillic acid. We found two minor peaks that could not be identified, which might represent lactobacillic acid, however, fatty acid methyl ester standards (Sigma) and GC-MS analysis were not conclusive in this respect. Addition of 8% (vol/vol) of ethanol to the growth medium increased the degree of fatty acid unsaturation, while the total lipid content markedly decreased (Fig. 3-6). This was mainly due to a strong decrease in C16:0 and an increase in the level of C16:1 (data not shown). Moreover, it was observed that two additional unsaturated short-chain fatty acids were present in cells grown in presence of ethanol (data not shown). The FA composition, the degree of unsaturation and the total amount of lipids were identical in control cells and in cells pre-incubated in 12% (vol/vol) of ethanol for 2 hours, irrespective of the presence of CAP (Fig. 3-6). Notably, these cells retained cF more efficiently (Fig. 3-2) and were more resistant to ethanol-induced membrane disordering (Fig. 3-3b).

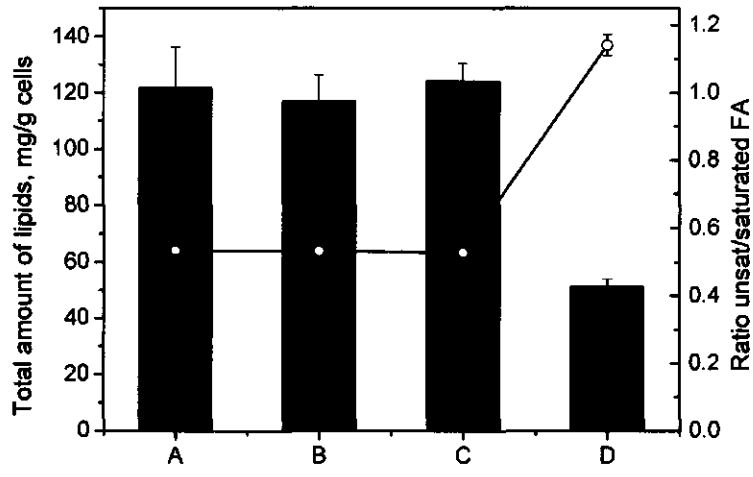

Figure 3-6 Membrane lipid composition of *O. oeni* cells. The total lipid content (bars) and the unsaturation/saturation ratio (○) where measured in control cells (A), cells exposed to 12% ethanol for 2 h in the presence (B) or the absence (C) of CAP, and cells grown in the presence of 8% ethanol (D).

3.5. Discussion

There is virtually universal agreement in the literature that biological membranes are the primary target of ethanol injury, and it is widely assumed that membrane physical properties and lipid composition are the main factors involved in ethanol tolerance (for a review see [25]). An important question arises as to the extent to which these aspects of membrane tolerance share common features. In the present paper, ethanol effects on the physical and chemical properties of the cytoplasmic membrane of *O. oeni* cells are described, also in relation to the acquired tolerance to ethanol. We found similar trends in the ethanol dependencies of membrane permeability and fluidity. This might be taken as preliminary evidence that these two membrane properties are ruled by a common mechanism: i.e. ethanol-induced leakiness is the consequence of membrane physical disordering, and resistance to the permeabilizing effect of ethanol would result from an adaptive increase in membrane order. However, there was no clear correlation between adjustment of membrane physical properties and changes in the unsaturated/saturated ratio of membrane fatty acids.

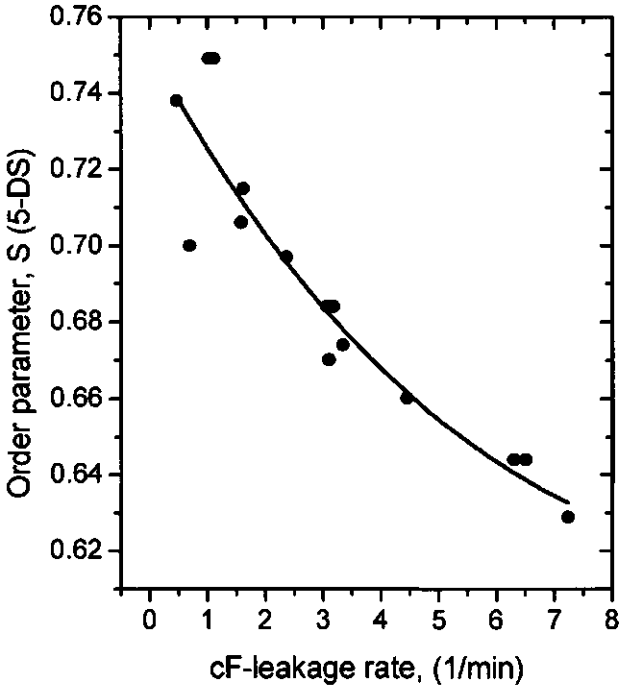

Figure 3-7 Correlation between S-values calculated from 5-DS spectra shown in Figs. 3-3 and 3-5 and cF-leakage-rate values from Fig. 3-2. The data relate to *O. oeni* cells grown without (control) or with 8% ethanol and cells pre-exposed for 2h to 12% ethanol in the presence or absence of CAP. The cells were exposed to 0, 8, 12 and 16% ethanol during ESR spectra recording and cF-efflux measurements.

From the ESR spectra of 5-DS it is clear that the fluidity of the cytoplasmic membrane in *O. oeni* cells instantaneously increases on addition of ethanol, in a concentration dependent manner. 5-DS allows the order to be examined at the upper methylene segment of the lipid hydrocarbon chains, i.e. close to the lipid-water interface. From the disturbance of this relatively immobile membrane segment it follows that ethanol partitions at least into the lipid-water interface. Ethanol also increased the freedom of motion of spin probes that are labelled along the fatty acid near the hydrophobic core of the membrane, but only for high concentrations of ethanol [20%(vol/vol)]. These results are in agreement with other studies showing that ethanol molecules reside mainly at the lipid-water interface near the lipid glycerol backbone of the hydrocarbon chains (14, 21).

The capacity for survival under what would normally be considered extremely adverse conditions, as those prevailing in wine, requires specific cellular strategies that are of fundamental importance for microbial life in such extreme environments. For optimal biological performance, membranes should be maintained in a fluid, liquid-crystalline state (22). We found that ethanol-adapted *O. oeni* cells were able to respond to the fluidizing effect of ethanol by increasing the order at the membrane lipid-water interface and decreasing permeability. These results are consistent with the theory that bacterial cells possess adaptation mechanisms to compensate for the accumulation of toxic amphiphilic compounds in their membranes (47). Interestingly, the readdition of 8% (vol/vol) of ethanol to cells grown in presence of 8% (vol/vol) of ethanol resulted in a membrane fluidity (at the position of the nitroxide label of 5-DS) that was similar to that in non-adapted cells in the absence of ethanol. This result implies that ethanol-induced adaptation in membrane fluidity is not only qualitatively, but also quantitatively consistent with the homeoviscous theory validated for bacteria by Sinensky (40).

Besides long-term ethanol-adaptation, achieved by cells growing in the presence of ethanol, cells need a means for rapid adjustment of ethanol-induced membrane disorder. Cells pre-exposed to 12% (vol/vol) of ethanol (2 h) acquired membrane ethanol-tolerance, although the plasma membranes from these cells were more disturbed by ethanol than those from cells that were grown in presence of 8% (vol/vol) of ethanol. The tolerance included adaptive changes in both order and permeability to negate the effect of ethanol. It has been suggested that ethanol-induced synthesis of stress proteins such as small hsps is associated with the enhanced ethanol tolerance in bacteria (23, 30). It was recently shown that small hsps interact with phospholipid bilayers and stabilize them (46). While such a mechanism may provide tolerance to *O. oeni* cells that were grown in the presence of 8% (vol/vol) of) ethanol, it is unlikely that a similar mechanism operates during the 2h pre-exposure to 12% (vol/vol) of ethanol, since the adaptation was not prevented in the presence of the protein synthesis inhibitor, CAP. This leaves the possibility that proteins or other compounds already existing in the cytoplasm are called upon to stabilize the cytoplasmic membrane e. g. an ethanol-induced increase in the affinity of cytoplasmic proteins toward membranes by increasing their hydrophobicity. These results appear to imply that mechanism(s) independent of *de novo* protein synthesis may be involved in the adaptive response of *O. oeni* cells to ethanol.

Figure 3-7 shows the correlation between S-values, calculated from 5-DS spectra, and cF-leakage rates in ethanol-treated cells grown in the presence or absence of 8% ethanol, or preexposed to 12%

(vol/vol) of ethanol for 2 h. Although no causal relationship between permeability and lipid order was established, Fig. 3-7 shows a strong negative correlation (r=-0.93) between these parameters, which suggests that cF-leakage rates are determined by the fluidity at the lipid-water interface. There are arguments that support a possible causal relationship. First, from membrane dynamics simulation it appeared that the order at the membrane region where the nitroxide moiety of 5-DS resides determines the ability of water and ions to diffuse across a membrane (43). Second, ethanol appears to reside at the lipid-water interface, which, together with the fore-mentioned permeability control at the upper methylene segment of the acyl chains, renders a direct link between ethanol-induced disorder and leakage plausible.

Adaptation of cells to ethanol would be most effective at the membrane lipid-water interface, where stress proteins are supposed to interact. Adaptive changes in the acyl chain composition may adjust membrane fluidity in the core region, which might be less effective in the case of ethanol. This is probably the reason for the less consistent picture that emerges from the literature concerning ethanol-induced changes in acyl chain composition (for a review, see [25]). In the present work we found a significant increase in the degree of unsaturation of the fatty in cells grown in presence of 8% (vol/vol) of ethanol, which is supposed to lead to a decrease in the gel-to-liquid-crystalline transition temperature and to increased fluidity. However, as determined by ESR, the cytoplasmic membranes in *O. oeni* cells grown in the presence of ethanol were much more rigid than those in control cells. The same phenomena were observed in *E. coli* cells, and liposomes made from the phospholipids of these cells displayed, as expected, increased fluidity (12). This observation could point to an important role for the protein content of membranes in regulating fluidity. Actually, we found that *O. oeni* cells grown in the presence of ethanol (8%) had reduced lipid content, i.e. half of that of the control cells. The decreased mobility of the membranes observed in these ethanol grown cells corroborates the idea that a selective decrease in the lipid content can increase the proportion of motionally hindered lipid molecules (26).

The membrane composition of cells pre-exposed to 12% (vol/vol) of ethanol for 2 h was identical to that of control cells. However, these pre-exposed cells showed decreased membrane permeability and disordering under ethanol-stress conditions. Thus, these results suggest that ethanol-adaptation at the membrane level, does not hold for changing membrane lipid composition. Moreover, the increased unsaturation level displayed by cells grown in the presence of ethanol, more than an effect of ethanol adaptation, as it has been suggested (15), is just a consequence of ethanol-induced inhibition of saturated FA synthesis (20, 22).

In this paper we conclude that *O. oeni* cells adjust their membrane permeability during ethanol-adaptation by decreasing fluidity at the lipid-water interface. Thus, we hypothesize that the physical state of membrane, rather than mere membrane composition, may preclude an important role during ethanol-adaptation by controlling other biological process, e.g. ATPase activity (9, 17) and transport systems (8, 48).

3.6. Acknowledgements

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3.7. References

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4. FLOW CYTOMETRIC ASSESSMENT OF BIOENERGETICS OF ETHANOL-STRESSED *OENOCOCCUS OENI* CELLS

4.1. Abstract

For an improved control of malolactic fermentation (MLF) in wine industry it is essential to understand the effect of ethanol on this pathway. In the present work, flow cytometry was used to investigate the effect of ethanol on bioenergetics of individual *Oenococcus oeni* cells. During the metabolism of glucose as the only carbon energy source *O. oeni* cells were not able to extrude efficiently carboxyfluorescein (cF), suggesting that the cF efflux in O. oeni takes place via a primary transport system, since ATP levels in these cells was very low. However, upon the addition of fructose together with glucose, cF efflux was strongly stimulated as well as during MLF. Flow cytometric analysis of cells pre-exposed to 12% (vol/vol) of ethanol for 1h, showed that two sub-populations could be distinguished, one of which being able to extrude cF during MLF, i.e. cells non-fluorescent and the other one that lost the ability to extrude actively the probe, i.e. cells cF-fluorescent. Addition of 12%(vol/vol) of ethanol promoted a decrease in the ATP pool in cells performing MLF and a rapid increase in the level of intracellular NAD(P)H in deenergized cells. The last phenomena was also observed upon the addition of an concentration of ethanol in the physiological range (100 mM), suggesting that a NAD(P)H/NAD(P)H unbalance is generated as a consequence of ethanol oxidation. Notably, cells grown in the presence of 8% (vol/vol) of ethanol were able to extrude cF during MLF after exposure to 12% (vol/vol) of ethanol for 1h. These cells displayed higher ATP and lower NAD(P)H pools under ethanol-stress conditions. The data give a coherent biochemical basis to understand the ethanol-induced inhibition of MLF in *O. oeni*. Moreover, we provide evidence that energy-dependent cF efflux can be used as an indicator of pmf generation and ATP synthesis in *O. oeni*.

Da Silveira, M. G., F. M. Rombouts and T. Abee. 2003. Flow cytometric assessment of bioenergetics of ethanol-stressed *Oenococcus oeni* cells. Appl. Environ. Microbiol. Submitted.

4.2. Introduction

The control of activity of lactic acid bacteria that carry out malolactic fermentation (MLF) is an important feature of the technology of modern commercial wine production (17). *Oenococcus oeni* is recognized as the principal microorganism responsible for the MLF (15), under stress conditions as those prevailing in wine. MLF consists of the decarboxylation of L-malic acid to L-lactic acid, leading to a decrease in the total acidity and improving the organoleptic properties and bacteriologic stability of wine (16).

The exact benefits of the MLF to the bacteria have been a matter of discussion among enologists for years. The lack of a pyruvate intermediate (7) and the fact that direct H^+ or Na^+ extrusion involving membrane-bound decarboxylase does not occur (12) prompted researchers to report that MLF serves a non-energy-yielding function. It was than suggested that MLF would contribute to the improved growth, by enabling a favorable pH that stimulate utilization of other carbon substrates (8, 24).

It is now well accepted that the MLF is a proton motive force-generating process that occurs in some lactic bacteria (18, 23, 25, 28). The decarboxylation of L-malic acid is catalyzed by a single enzyme, called malolactic enzyme (MLE), which is NAD^+ and Mn^{2+} dependent. In *O. oeni* the proton motive force (pmf) is generated during MLF as a consequence of the electrogenic transport of monoprotonated malate and concomitant consumption of a proton in the cytoplasm during its decarboxylation (25, 27). The pmf generated is of sufficient magnitude to drive ATP synthesis by the H^+-ATPase (9, 14, 28).

Although it was shown that the presence of a pmf is not essential for growth under certain conditions (13), it becomes important as the external pH decreases and the concentration of essential nutrients that are transported by secondary transport systems becomes low. Since, these conditions are generally found in wine where ethanol promotes the passive influx of proton (10), maintenance of pmf generating capacity is a crucial feature in the performance of *O. oeni* in wine.

For an improved control of MLF in wine industry it is essential to understand the effect of ethanol on this pathway and its impact on bioenergetics. ATP synthesis and substrate degradation rate are classical methods to evaluate metabolic activity. However, both techniques involve bulk measurements and assume that all the cells will contribute equally to the global performance of MLF. The use of fluorescent probes in combination with flow cytometry (FCM), allows the discrimination of cells in different physiological states (11). In this study, we used the esterified pre-fluorochrome carboxyfluorescein diacetate (cFDA), which is converted to carboxyfluorescein (cF) by functional cytoplasmic enzymes i.e. esterases. cF (the green fluorescent form) is negatively charged at physiological pH, and consequently will accumulate inside cells with an intact cytoplasmic membrane. However, cF can be actively extruded from cells by transport systems (3, 5, 22), which represents a powerful tool to assess the metabolism capacity of the cells. The design of our approach is represented in figure 4-1. Our results provide evidence that active extrusion of cF

can be used to assess malolactic activity in *O. oeni*. Multiparameter FCM analysis of control, ethanol-stressed and ethanol-adapted *O. oeni* cells allows assessment of population heterogeneity, i.e. different sub-populations of *O. oeni* with respect to their ethanol tolerance can be readily identified, which may provide tools for optimization of MLF in wine. A biochemical basis for ethanol-induced inhibition of malolactic activity is also presented.

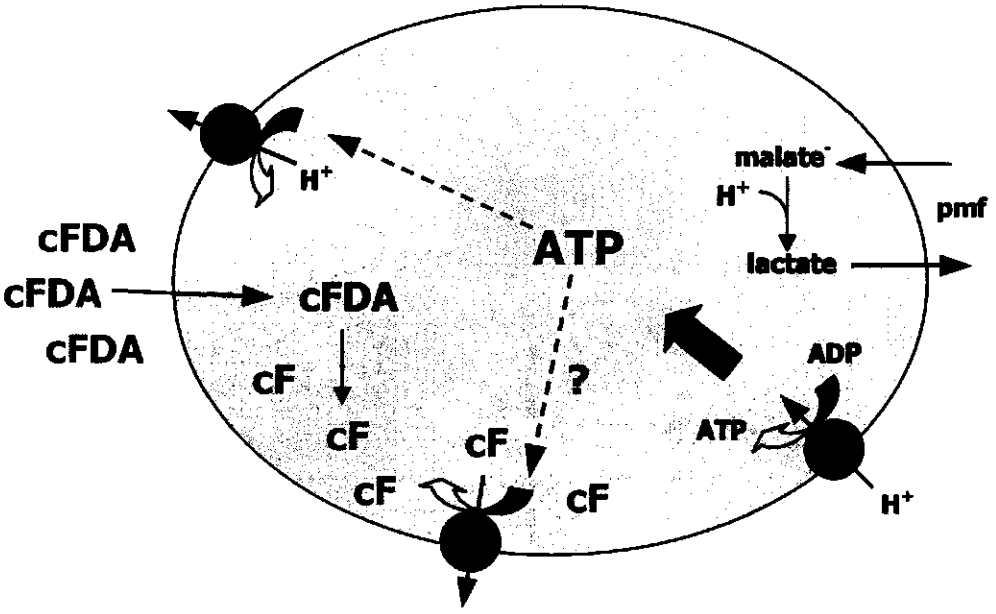

Figure 4-1 Schematic figure of cF labeling and efflux in *O. oeni* cells performing MLF. The question marks represent our hypothesis: *O. oeni* possess a transport system with affinity for cF; cF extrusion is ATP-dependent.

4.3. Materials and Methods

Bacterial strain and growth conditions. *Oenococcus oeni* GM (Microlife Technics, Sarasota, Fl.) was cultured at 30°C in FT80 medium (pH 4.5) (6) modified by the omission of Tween 80, containing 10 g of DL-malic acid per liter. Glucose and fructose were autoclaved separately and added to the medium just before inoculation, at a final concentration of 2 and 8 g per liter, respectively. Early stationary phase cultures were diluted 100-fold in fresh medium, incubated for 24 h, and then used to obtain 1% inoculated cultures. In the adaptation experiments the final culture medium was supplemented with 8% (vol/vol) ethanol.

Stress conditions. *O. oeni* was cultured in normal conditions or in presence of ethanol and harvested by centrifugation at exponential phase (20 h and 40h respectively). Cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.0) with or without 12% (vol/vol) of ethanol and incubated at 30°C for one hour. Cells were recovered by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 5.

Loading of cells with carboxyfluorescein (cF). Cells were harvested at the end of the exponential growth phase (OD_{600}≈0.4) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 20. The cells were de-energized with 2-deoxyglucose (a glucose analogue of which the phosphorylation results in depletion of ATP) at a final concentration of 2 mM, by incubation at room temperature during 30 min. The cells were washed and re-suspended in 50 mM KPi buffer (pH 7.0) to an OD_{600} of 20 for fluorimetrical analyses or to an OD_{600} of 5 for flow cytometric analyses. A stock solution of 2.3 mg of 5(6)-carboxyfluorescein diacetate (cFDA) (Molecular probes, Eugene, Oregon) per ml was prepared in acetone and stored at -20°C in the dark. cFDA was added to a concentration of 50 µM to the cell suspension and the mixture incubated at 30°C for 15 min (or 60 min for cells pre-grown in presence of 8% (vol/vol) ethanol and stressed with 12% (vol/vol) ethanol). Immediately after labeling, the cells were spun down, washed once, and resuspended in 50 mM KPi (pH 7.0) to an OD_{600} of 2.0 for fluorimetrical analyses.

Measurement of cF efflux. cF-loaded cells were washed twice and re-suspended in 50 mM KPi buffer (pH 7.0) to a final OD_{600} of 2.0. At time zero, cell suspensions were placed in a waterbath at 30°C and incubated without and with energetic source (glucose 10 g/l, glucose 2g/l plus 8 g/l fructose and malic acid 45 mM). Samples (200 µl) were withdrawn at specific time points and immediately centrifuged to remove the cells. To measure the cF labeling capacity, labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed by centrifugation. The fluorescence of the supernatant was measured fluorimetrically (excitation at 490 ± 5 nm and emission at 515 ± 5 nm), with a Perkin-Elmer LS 50B luminescence spectrometer. From the fluorescence of the supernatants and the total labeling capacity, the intracellular concentrations of cF at the sampling points were calculated. For flow cytometry assays a suspension of *O. oeni* labeled cells (OD_{600}=5) was diluted 1000-fold in 50 mM KPi (pH 7.0 for sugar co-metabolism assay and 5.5 for malic acid assay) to a final cell concentration of approximately 10^6 cells per ml in the absence and presence of energetic source (glucose 2g/l plus 8 g/l fructose and malic acid 45 mM). At time zero, the cells were placed in a waterbath at 30°C. Time series were made by taking 100 µl aliquots that were diluted with 50 mM KPi (pH 7.0 or 5.5) to a final volume of 1 ml and immediately analyzed.

Flow cytometric analysis. The FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used for single-cell light scattering and fluorescence measurements. The samples were illuminated with a 15 mW 488 nm, air-cooled Argon-ion laser, and fluorescence emission was detected at 530 nm for cF and at >670 nm for PI. List mode data from approximately 5000

cells were collected and processed by using the CELLQuest program (version 3.1f; Becton Dickinson). Photomultiplier amplifier gains were set in the logarithmic mode for both light scattering and fluorescence. A combination of forward (FSC) and side scatter (SSC) was used to discriminate bacteria from background. Side angle light scattering, triggered light scattering and fluorescence with the threshold limit set to 200 channels, in order to reduce background noise. Data were analyzed with the WinMDI program (version 2.8: Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia [http://jcsmr.anu.edu.au]).

ATP concentration. ATP concentration measurements were made under the same conditions as cF efflux measurements. For the ATP synthesis inhibition we used cells energized with malic acid (45 mM) in non-growing conditions (50 mM KPi pH 3.5) as control and ionophores (valinomycin 2.8 μM plus nigericin 7.5 μM) and ethanol [12%(vol/vol)] were added after 10 minutes. Samples (20 μl) were withdrawn and mixed with 80 μl of diethyl sulfoxid and after 5 minutes diluted with 5 ml of de-ionized water. The ATP content was measured in an M 2500 biocounter (Lumac, Landgraaf, The Netherlands), with the Lumac luciferin/luciferase assay.

Fluorescent measurement of NAD(P)H. NAD(P)H concentration measurements were made under the same conditions as cF loading assays. The non-adapted and adapted cells were re-suspended in 50 mM KPi buffer (pH 7.0) to an OD_{600} of 0.3. The levels of intracellular reduced nicotinamide nucleotides were monitored measured fluorimetrically (excitation at 350 ± 5 nm and emission at 440 ± 5 nm), with a Perkin-Elmer LS 50B luminescence spectrometer. The experiments were carried out in a thermostatted cuvette at 30°C with stirring and ethanol was added to a final concentration of 12% (2 M) and 100 mM (0.46%). Arbitrary units of fluorescence intensity were converted to molar amounts of NADPH, calibrating the instrument with solutions of known NADPH concentration.

Malic acid consumption. Cells were harvested at the end of the exponential growth phase (OD_{600}≈0.4) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 20. Cells were de-energized with 2-deoxyglucose (at a final concentration 2 mM) by incubation at room temperature for 30 min. The cells were washed and resuspended in 50 mM KPi buffer (pH 3.5 adjusted with HCl) to an OD_{600} of 2. At time zero cell suspensions were placed in a water bath at 30°C and incubated with 3 g/l of L-malic acid (pH 3.5). Samples (50 μl) were withdrawn at time intervals and immediately centrifuged to remove the cells. The supernatants (20 μl) were placed into water-bath at 80°C for 15 min to stop the enzymatic reactions. The L-malate concentrations were assayed enzymatically using Boehringer Mannheim kits and procedures.

Additional Analytical Methods. Cellular dry weight was determined by filtering 80 ml of the cell suspensions through pre-weighed polyethylene filters, with a porosity of 0.22 μm, and dried at 100°C in an oven until a constant weight was reached. As a control the dry weight of the same volume of phosphate buffer was also determined. Protein was assayed by the method of Lowry et al. (1951).

4.4. Results

cF extrusion at the population level. In order to ensure that *O. oeni* cells are able to extrude actively cF, de-energized cells of *O. oeni* were loaded with cF and subsequently extrusion of the probe was studied in the presence of different energy sources by measuring spectrofluorimetrically the amount of cF released in the supernatant of the cell suspension. *O. oeni* cells retain cF when not energized, however, a gradual release of cF in the supernatant was observed at a rate of 0.5% per min (Fig. 4-2). Cells energized with glucose alone were not able to extrude efficiently cF. These cells lost the probe at a rate similar to that of de-energized cells (0,8% per min). However, upon the addition of fructose together with glucose, cF efflux was strongly stimulated, i.e. 50% in 8 min ($t_{1/2}$). In cells energized with malic acid the same $t_{1/2}$ was observed, although, after 10 min the extrusion of cF was less efficient in these cells, i.e. after 25 min the cells still retained 30% compared with 15% in cells metabolizing glucose and fructose (Fig. 4-2). These results show that *O. oeni* possesses a transport system for cF, suggesting that the inability to extrude cF when cells were energized with glucose alone is associated with a lack of energy generation.

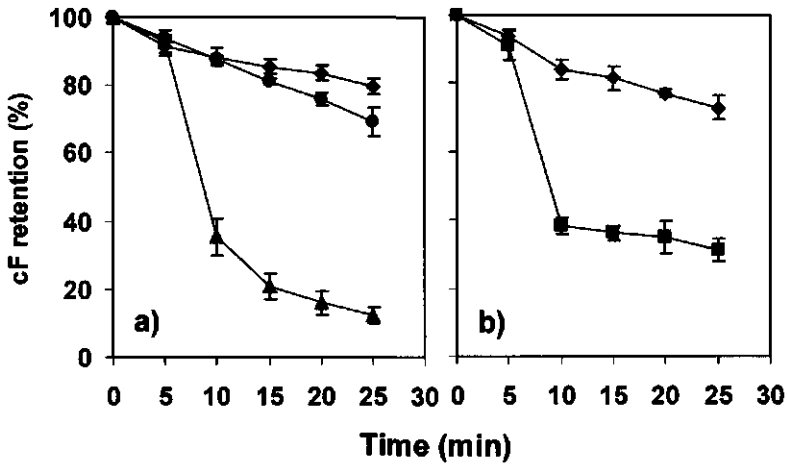

Figure 4-2 cF efflux in *O. oeni* cells loaded with cF by incubation at 30°C with 50 µM cFDA. The efflux of cF was measured at 30°C in 50 mM KPi buffer pH 7.0 (a) and at pH 5.5 (b) in de-energized cells (♦), cells energized with Glucose (●); with glucose plus fructose (▲) and with malic acid (■).

ATP synthesis. To address this hypothesis, ATP synthesis was monitored in cells metabolizing different carbon substrates. From our results it is evident that *O. oeni* cells are not able to synthesize ATP with glucose as the only carbon and energy source (Fig. 4-3). This observation indicates that cF efflux is associated with the intracellular ATP levels, confirming previous observation that cF extrusion is most likely mediated by an ATP-driven transport system (5, 22). The rates of ATP synthesis were the same in cells performing glucose-fructose cometabolism and in cells performing MLF. ATP synthesis by malolactic activity was performed at

optimal conditions i.e. pH 3,5 and at 45 mM L-malate (28). However, for cF efflux assays we had to use a higher pH (5,5) to avoid extensive protonation of cF and subsequent passive efflux from the cell. This can explain the difference observed for cF extrusion in cells energized with sugar and malate. Moreover, the internal pH is not the same in cells cometabolizing glucose-fructose and in cells performing MLF. Since, cF fluorescence intensity is pH dependent (4), it is possible that the differences observed in the values of cF retention in both situations didn't reflect just only a concentration but also a fluorescence intensity effect.

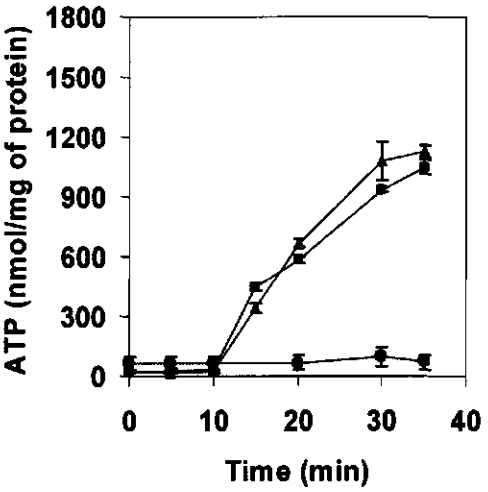

Figure 4-3 ATP synthesis during metabolism of different carbon sources. *O. oeni* cells were diluted 10-fold in 50 mM KPi buffer pH 7.0 (except for malic acid that was 3.5) to a final OD_{600} of 2. Cells suspension were incubated at 30°C and glucose (●); glucose plus fructose (▲) and malic acid (■) were added after 10 minutes. At times indicated samples were withdrawn and analyzed for ATP content.

FCM assessment of cF extrusion by malate-energized cells. Flow cytometry was used to analyze bioenergetics at the level of individual cells. In the FCM analyses, the *O. oeni* cells were easily detected by their light scatter. In the dotplot of the FSC and the SSC a region was defined that comprised the cell population. Interfering particles that also had a SSC above the threshold, but were not in the delineated region were thus disregarded.

In cell suspensions of *O. oeni* grown without ethanol, 96% of the population could be labeled with cF (Fig. 4-4a). These cells retained the probe efficiently at pH 5.5 in the absence of an energy source, but upon the addition of malate efflux was strongly induced, i.e. after 5 min almost all the cells had lost the cF (95%). When cells were exposed to 12% (vol/vol) ethanol for 1 hour, they appeared to have lost the capacity to retain the probe at pH 5.5 in the absence of an energy source, suggesting membrane damage. In this condition the intracellular pH most likely decreases resulting in protonation of the probe and subsequent leakage from the cells by passive diffusion. After 15 min less than 40% of the cells were considered cF fluorescent. Moreover, these ethanol-stressed cells were not able to extrude cF. Single-parameter histograms of cF-stained cells showed that after 2 minutes two sub-populations could be distinguished, one of which being able to extrude cF and the other one giving a peak in the green fluorescence histogram (Fig. 4-5). After this time point, this sub-population lost cF by passive diffusion

indicated by the shift of the peak to the left (Fig. 4-5). However, this effect is not as evident as in control cells, since cells energized with malic acid were probably able to keep the internal pH by using the ATP driven from malolactic activity. FCM analysis thus revealed that the population of *O. oeni* is heterogeneous with regard to ethanol resistance based on maintenance of the cF-extrusion capacity.

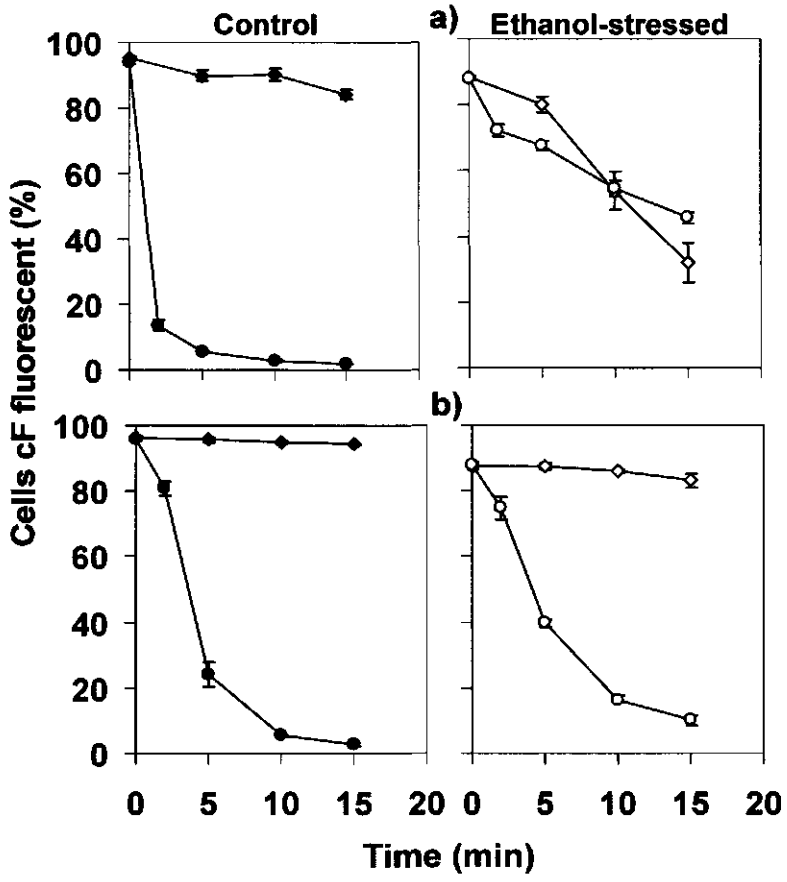

Figure 4-4 Flowcytometric analysis of cF efflux in *O. oeni* cells loaded with cF by incubation at 30°C with 50 μM cFDA. The efflux of cF was measured non-stressed cells (closed symbols) and in cells pre-exposed to 12% (vol/vol) ethanol for 1 hour (open symbols) in de-energized cells (♦) and in cells energized with malic acid (●). cF extrusion was assessed by flow cytometry in non-adapted (a) and in ethanol-adapted cells (b).

Notably, cells grown in the presence of 8% (vol/vol) ethanol were able to retain the probe efficiently in the absence of energy source and showed active extrusion of cF upon initiation of MLF (Fig. 4-4b).

Interestingly, *O. oeni* cells that were not exposed previously to ethanol were less efficient extruding cF, i.e. after 5 min 37% the cells were still fluorescent compared to 5% in non-adapted cells.

Ethanol-stressed cells were also tested for their labeling capacity, and it was found that stressing the cells with 12% (vol/vol) ethanol for 1 hour resulted in a sub-population that was not labeled with cF in both adapted (11%) and non-adapted cells (12%).

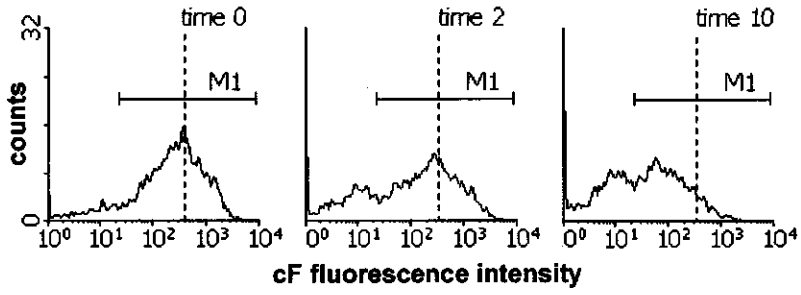

Figure 4-5 Single-parameter histograms of cF-loaded *O. oeni* cells pre-exposed to 12% (vol/vol) ethanol for 1 hour. Cells were incubated with 50 μM cFDA in KPi buffer (pH 7) at 30°C. Subsequently, cells were energized with malic acid (45 mM) and tested for cF extrusion. In the FCM analysis of *O. oeni* cells the labeled population gave a peak in the green fluorescence histogram (M_1), which was resolved from the signal of non-labeled cells.

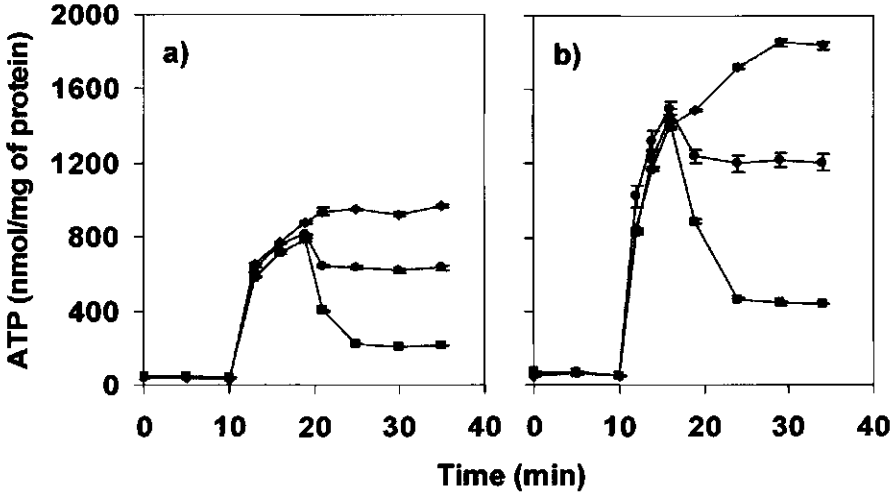

Figure 4-6 ATP synthesis during malolactic activity. *O. oeni* cells were diluted 10-fold in 50 mM KPi buffer pH 3.5 to a final OD_{600} of 2. Cell suspensions were incubated at 30°C and malic acid was added after 10 minutes. The arrow indicates the time of addition of 12% (vol/vol) ethanol (■); nigericin plus valinomycin (●); or no addition (♦). ATP content was determined in non-adapted (a) and in ethanol-adapted cells (b).

ATP synthesis inhibition. The previous results showed that ethanol-induced membrane damage is associated with the loss of ability to extrude cF actively, suggesting a correlation between pmf dissipation, inhibition of ATP synthesis and, consequently, blocking of active cF-extrusion. Addition of valinomycin and nigericin, resulted in a complete inhibition of ATP synthesis during malolactic fermentation (Fig. 4-6a). So, this result together with the previous observation that pmf is dissipated by valinomycin and nigericin in *O. oeni* (28), confirms our assumption that ATP synthesis during malolactic fermentation is strictly pmf-dependent. However, addition of 12% (vol/vol) ethanol not only blocked ATP synthesis but

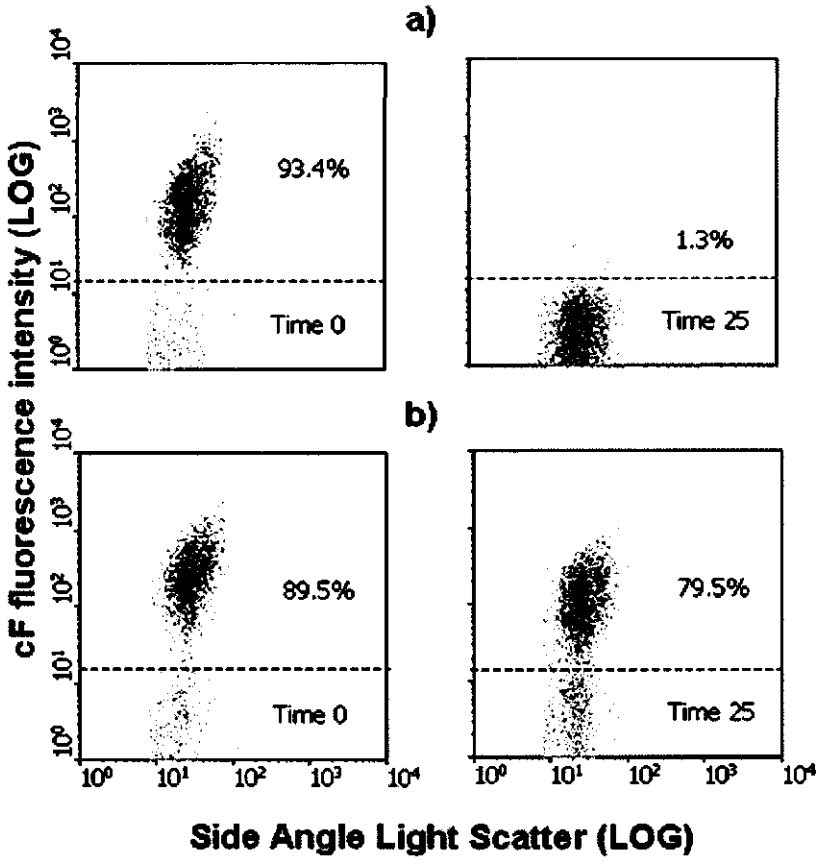

Figure 4-7 Flowcytometric analysis of cF efflux in *O. oeni* cells. Two-parameter dot plots are shown of de-energized cF-loaded cells incubated with glucose (2g/l) and fructose (8g/l), at 30°C in KPi buffer (pH 7). Cell populations were analyzed immediately after sugar addition and after 25 min of incubation. cF extrusion was assessed by fluorescence decline in control cells (a) and cells stressed with 12% (vol/vol) of ethanol for 1 hour (b).

promoted as well a decrease in the ATP pool, suggesting that other inhibitory mechanisms, other than pmf-dissipation, are involved. The same phenomenon was observed in ethanol-adapted cells, however in these cells the rate of ATP synthesis was much higher (Fig. 4-6b). Thus, the increased intracellular ATP levels available are now much higher, which may be advantageous for the cells under stress conditions.

FCM assessment of cF extrusion by sugar-energized cells. When *O. oeni* cells were incubated in 50 mM KPi buffer (pH 7.0) without a carbon source they retained the probe efficiently (data not shown). When cF-loaded cells were incubated with glucose plus fructose for 25 min the probe was extruded actively, as shown by the decrease in the fluorescence intensity in the 515 nm fluorescence/side scatter dot plots (Fig. 4-7a). Less than 2% of the cells were considered cF fluorescent. In cells pre-exposed to 12% (vol/vol) ethanol for 1 hour and energized with glucose plus fructose for 25 min, two sub-populations could be distinguished (Fig. 4-7b) one of which being able to extrude the probe and the other one that lost this ability (79.5%). These results support the hypothesis that other mechanisms than just pmf dissipation affect active extrusion of cF in ethanol-stressed cells, since ATP synthesis during the co-metabolism of glucose and fructose is pmf independent.

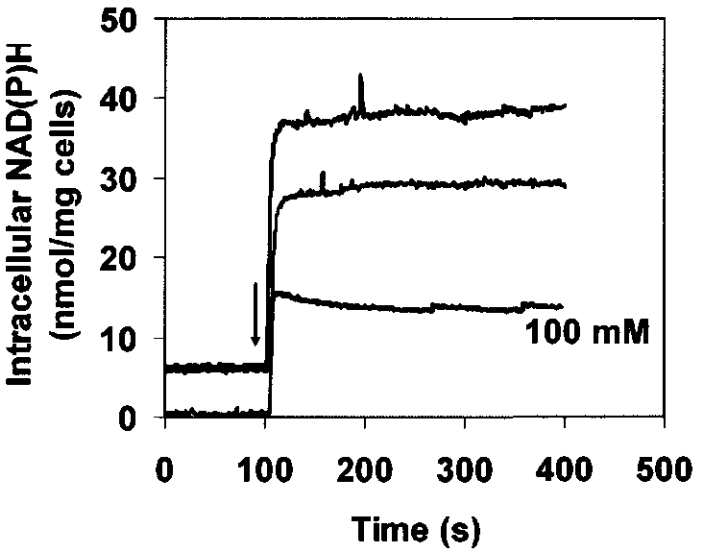

Figure 4-8 Time course for the change in the levels of intracellular reduced nicotinamide nucleotides [NAD(P)H]. Cell suspensions of *O. oeni*, previously de-energized were incubate at 30°C in KPi buffer (pH 7). At the time indicated by the arrow 12% (vol/vol) of ethanol (2 M) was added to non-adapted cells (——) and ethanol-adapted cells (——). Intrecellular concentrations of NAD(P)H were also assessed in non-adapted cells in the presence of a physiological concentration of ethanol (100 mM) .

NAD(P)H levels. NAD^+ is an essential cofactor for the malolactic enzyme activity and its reduced form has been reported to inhibit malolactic activity (1, 30, 31). Fluorescence measurements in whole cells were performed to evaluate the accumulation of reduced nicotinamide adenine nucleotides in *O. oeni* (Fig. 4-8). Following the addition of ethanol (2M), the level of intracellular NAD(P)H increased rapidly and a steady state was reached immediately (Fig. 4-8). These results suggest that ethanol induces a $NAD(P)^+$/NAD(P)H unbalance in *O. oeni* cells. The same phenomenon is observed in ethanol-adapted cells, however in these cells the amplitude of the increase was lower (29 nmol/mg cells compared to 33

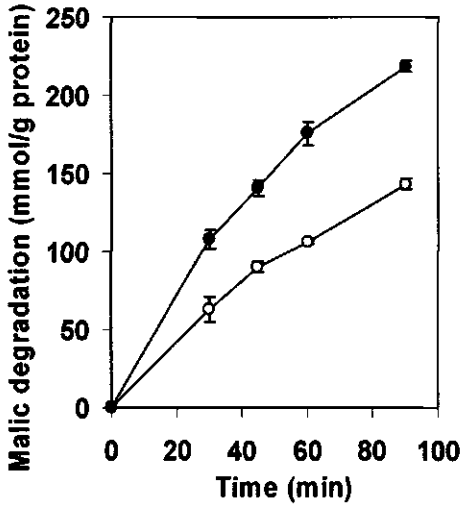

Figure 4-9 Malolactic activity in whole cells of *O. oeni*, monitored by malate consumption. The experiments were performed at pH 3.5 and at 45 mM L-malate in control cells (open circles) and cells grown in the presence of 8% (vol/vol) of ethanol (close circles).

nmol/mg in control cells). Moreover, almost no NAD(P)H was detected in the ethanol-adapted cells before ethanol addition, while in control cells it was 6 nmol/mg cells. NAD(P)H generation was also evaluated for ethanol concentrations in the physiological range. The direct fluorescence measurements of NAD(P)H in the presence of 100 mM of ethanol resulted in an intracellular increase of NAD(P)H of 9 nmol/mg cells. This observation suggests that *O. oeni* is able to metabolize ethanol.

Malolactic activity. Malolactic activity was determined as the rate of malic acid degradation. The experiments were performed at pH 3.5 and at 45 mM L-malate, since previous results have shown that 3.5 is the optimal pH for malolactic fermentation in this organism and 45 mM is a saturating concentration of substrate (28). Cells grown in the presence of 8% (vol/vol) of ethanol can degrade malate more efficiently than control cells (Fig. 4-9). However, these ethanol-grown cells were less efficient extruding cF than control cells, suggesting that the cF-efflux system is less efficient in ethanol-grown cells probably as a consequence of the high rigidity of the plasma membrane of these cells (chapter 3).

4.5. Discussion

Although flow cytometry is a promising tool, its application to monitor fermentation processes is still limited. Given the speed of analysis and the diversity of cell characteristics that can be measured, flow cytometry can be applied for online-monitoring providing detailed information about fermentation processes. Thus, it represents a sophisticated tool that allows direct regulation of the process conditions in order to optimize the production. Therefore, it was our approach to evaluate the validity of the fluorescence assays as indicators for pmf generation in *O. oeni* by comparing the fluorescence-related parameters with ATP-producing capacity and malolactic activity after ethanol stress and/or adaptation.

Labeled *O. oeni* cells actively extrude the accumulated cF upon energizing with malic acid, i.e. after 5 minutes less than 5% of the cells were still fluorescent. The rapid efflux indicates a high affinity of the extrusion system for cF. Of the known extrusion systems only multidrug resistance transport systems have demonstrated broad substrate ranges, and since cF doesn't resemble any naturally occurring compound, we can expect that in *O. oeni* cF is extruded by such an extrusion system as reported for BCECF in *Lactococcus lactis* (22). Bunthoff et al (1999) observed that *L. lactis* cells actively extrude cF after dissipation of the pmf by valinomycin and nigericin, suggesting that the cF efflux takes place via a primary transport system, which is most probably ATP dependent. The inability of *O. oeni* cells to extrude cF during metabolism of glucose alone, support this hypothesis, since ATP levels in these cells was very low. Previous studies concerning the metabolism of glucose by *O. oeni* have shown that the activity of acetaldehyde dehydrogenase is very low compared to the activity of NAD(P)H-forming enzymes in the early steps of glucose metabolism, preventing efficient NAD(P)H disposal during glycolysis, leading to a high intracellular concentration of NAD(P)H (32). Consequently, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are inhibited, which in turn results in the inhibition of glucose metabolism. Moreover, the efficient extrusion of cF observed in *O. oeni* cells during glucose-fructose cometabolism provides further evidence that cF extrusion is most likely mediated by an ATP-driven transport system. When an external electron acceptor, such as fructose, was added the ATP synthesis was strongly stimulated. Fructose is partially converted to mannitol via mannitol dehydrogenase (29, 33), providing an extra route for the reoxidation of NAD(P)H. Thus, in cells cometabolizing glucose-fructose, the NAD(P)H/NAD(P)$^+$ ratio decreases, with concomitant draining of accumulated intermediate compounds in the metabolism of glucose and consequent relief of inhibition of its metabolism (21).

O. oeni cells exposed to 12% (vol/vol) ethanol for 1 hour lost the capacity to extrude efficiently the cF, which might reflect the loss of the capacity for metabolic energy generation. During malate metabolism the ATP synthesis is dependent on the magnitude of the pmf generated by malate transport and decarboxylation. So, the inability of *O. oeni* to extrude the probe after ethanol stress is associated with the absence of pmf, as a result of pmf dissipation and/or inhibition of malolactic enzyme and therefore failure of pmf generation. During malolactic fermentation addition of valinomycin and nigericin resulted in a complete inhibition of ATP synthesis, as a consequence of the pmf dissipation as observed before (28). Under the

same experimental conditions, the addition of 12% (vol/vol) ethanol promoted a significant decrease of the ATP pool, suggesting that also other inhibitory effects are involved. Moreover, in cells pre-exposed to 12% (vol/vol) ethanol for 1 hour and energized with glucose plus fructose instead of malic acid, 80% of the population lost the ability to extrude the probe efficiently. These results are in line with the idea that other mechanisms than pmf dissipation are involved in ethanol-induced cF-efflux inhibition, since during the glucose-fructose co-metabolism ATP synthesis is pmf independent, i.e. is generated by substrate-level phosphorylation.

These results triggered further effort to understand the biochemical principles underlying this inhibition effect. It is known that the ratio NAD(P)H/ $NAD(P)^+$ plays an important regulatory role in multiple interlocked pathways in *O. oeni* and consequently in the global metabolism (20, 21, 32). Addition of 12% (vol/vol) ethanol to de-energized cells of *O. oeni* increased strongly the intracellular levels of NAD(P)H. In *O. oeni* the $NAD(P)^+$ produced during the first steps of the heterolactic fermentation of sugars is partially reoxidized by the conversion of acetyl phosphate to ethanol (20, 26). It seems reasonable to assume that if this reaction proceeds in the reverse direction, i.e. oxidation of ethanol to acetaldehyde, an increase in NAD(P)H pool will occur, since the oxidation of ethanol to acetaldehyde consumes $NAD(P)^+$ and produces NAD(P)H. This possibility was pursued by evaluating NAD(P)H generation in the presence of concentrations of ethanol within the physiological range. The direct fluorescence measurements of NAD(P)H in the presence of 100 mM of ethanol resulted in an intracellular increase of NAD(P)H of 9 nmol/mg cells (Fig. 4-8). This observation is in full agreement with the proposed hypothesis. Although NAD(P)H is unable to inhibit the malolactic activity of the pure enzyme, in whole cells a significant inhibitory effect is observed which can be explained by the conversion of NADPH to NADH by transdehydrogenase activity (21). Several studies have reported the inhibition of the malolactic enzyme by NADH (1, 30, 31). NAD^+ is an essential cofactor for the enzyme activity, NADH being a closely related compound, competes efficiently for the binding site of NAD^+, thereby causing inhibition.

Previously, we observed that ethanol induced *O. oeni* membrane damage, stimulating the leakage of intracellular compounds absorbing at 260 nm after 5 minutes of exposure. Typical compounds absorbing at 260 nm include NAD^+, NADH, and AMP (16). Although, it is expected that the nonspecific leakage of intracellular material will affect the balance between $NAD(P)^+$ and NAD(P)H, in the present work we observed that the intracellular increase of NAD(P)H occur instantaneously after ethanol addition, so, when the ethanol-induced leakage takes place the unbalance between $NAD(P)^+$ and NAD(P)H is already installed.

Growing cells in the presence of 8% (vol/vol) ethanol appeared to suppress the ethanol-induced inhibition of cF extrusion, given that after being exposed to 12% (vol/vol) ethanol for 1 hour these ethanol-adapted cells were as efficient as non-stressed cells extruding actively the probe. Interestingly, addition of 12% (vol/vol) ethanol to adapted cells resulted in an intracellular level of NAD(P)H 10 nmol/mg cells lower than in control cells. This observation provides further evidence that inhibition of malolactic activity by

ethanol can be attributed to an indirect effect of ethanol on the malolactic enzyme, by increasing the intracellular level of NAD(P)H. Our explanation for the inhibition of MLF by ethanol is outlined in the figure 4-9. Malic acid degradation is much more efficient in ethanol-adapted cells, which present lower intracellular levels of NAD(P)H. These results justify the high levels of ATP synthesis observed in cells grown in the presence of ethanol.

It would be of major interest to find out if the subpopulation of cells that are able to extrude cF also have lower levels of NAD(P)H than the cells that lost the ability of extruding actively the probe after being exposed to 12% (vol/vol) ethanol for 1 hour. However, an important drawback of FCM sorting is that the cell concentration has to be low, so that, on average, every three droplets will contain a cell. Thus, the number of cells that we can collect is far from enough to measure the intracellular pool of NAD(P)H. The heterogeneity found in the *O. oeni* population under ethanol-stress conditions is most probably physiological (2), which invalidates the possibility of growing the cells belonging to different subpopulations in order to obtain enough cells to check for intracellular pools of NAD(P)H.

In previous work we observed that ethanol compromises the integrity of the cytoplasmic membrane leading to the passive influx of protons and promoting the leakage of intracellular compounds absorbing at 260 nm (10). Conceivably, nonspecific leakage of intracellular material will include manganese, an important cofactor of malolactic enzyme. So, the ethanol-induced membrane damage coupled with increase of intracellular pools of NAD(P)H, offers an explanation for the inhibitory effects of ethanol on *O. oeni* metabolism.

Besides the fundamental interest underlying the elucidation of ethanol-induced inhibition of malolactic fermentation achieved in this work, our results may provide useful metabolic directives for improvement of starter cultures. Formerly, we set a rapid method to assess membrane integrity of *O. oeni* cells, of major interest to the starter culture industries as an indicator of the physiological state of the individual *O. oeni* cells. In the present work we provide a rapid method to assess the bioenergetics of individual *O. oeni* cells, which represents a powerful technique to the wine industry allowing online monitorization of malolactic activity at the single cell level.

4.6. Acknowledgements

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5. EFFECT OF ADAPTATION TO ETHANOL ON CYTOPLASMIC AND MEMBRANE PROTEIN PROFILES OF *OENOCOCCUS OENI*

5.1. Abstract

The practical application of commercial malolactic starter cultures of *Oenococcus oeni* surviving direct inoculation in wine requires insight into mechanisms of ethanol toxicity and of acquired ethanol tolerance in this organism. Therefore, the site-specific location of proteins involved in ethanol adaptation including cytoplasmic, membrane-associated and integral-membrane proteins was investigated. Ethanol triggers alterations in protein patterns of *O. oeni* cells stressed with 12% of ethanol for 1 hour and that of cells grown in the presence of 8% of ethanol. Levels of inosine-5'-monophosphate dehydrogenase (IMDH) and phosphogluconate dehydrogenase (PGDH) that generate reduced nicotinamide nucleotides, were decreased during growth in the presence of ethanol, while glutathione reductase (GR) that consumes NADPH, was induced suggesting that maintenance of the redox balance plays an important role in ethanol-adaptation. PTS components of mannose PTS including phosphocarrier protein HPr (HPr) and the EII^{Man}, were lacking in ethanol-adapted cells providing strong evidence that mannose PTS is absent in ethanol-adapted cells, and this represents a metabolic advantage to *O. oeni* cells during malolactic fermentation. In cells grown in the presence of ethanol a large increase in the number of membrane-associated proteins was observed. Interestingly, two of these proteins, dTDT-glucose 4,6 dehydratase and D-alanine:D-alanine ligase, are known to be involved in cell wall biosynthesis. Using a proteomic approach we provide evidence for an active ethanol adaptation response of *O. oeni* at the cytoplasmic and membrane protein level.

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5.2. Introduction

Malolactic bacteria are lactic acid bacteria that are able to carry out malolactic fermentation (MLF). The control of their activity is an important aspect of the technology of commercial wine production. MLF consists of the decarboxylation of L-malic acid to L-lactic acid, which decreases total acidity and improves the stability and quality of wine. *Oenococcus oeni* is recognized as the principal microorganism responsible for MLF under stress conditions, such as those prevailing in wine. However, inoculation of *O. oeni* starter cultures directly into wine leads to significant cell mortality and, consequently, failure of MLF. For an improved control of MLF in wine industry it is essential to understand the mechanisms involved in ethanol stress and tolerance (for a review in MLF see [28]).

We have previously examined the effects of ethanol in *O. oeni* cells and have shown that ethanol acts as a disordering agent of the *O. oeni* cytoplasmic membrane (7, 9) and negatively affects metabolic activity (8). The variety of inhibitory consequences of ethanol exposures makes assignment of primary targets problematic. In this paper, we examined not only the composition of cytoplasmic proteins of *O. oeni* cells but also that of membrane proteins in cells grown in the presence of ethanol. Membrane proteins may be associated with the membrane (extrinsic or peripheral membrane proteins) or be integrated in the membrane (intrinsic or integral membrane proteins). Integral membrane proteins contain one or more hydrophobic segments of the polypeptide chain (consisting predominantly of hydrophobic amino acids), which are able to span the membrane, sometimes repeatedly, and therefore are called membrane-anchoring domains (46). Membrane associated proteins are generally bound to the membrane by protein-protein interaction (34), but may also include lipoproteins attached covalently to the membrane by acetylation.

One widely studied aspect of the ethanol-stress response is the modification of the cellular protein composition, including the so-called heat shock proteins (hsps) (20, 30, 42). Recently, Bourdineaud et al (2) found that the *O. oeni ftsH* gene, encoding a protease belonging to the ATP binding cassette protein superfamily, was stress-responsive, since its expression increased at high temperature and under osmotic shock. Expression of the *O. oeni ftsH* gene in *E. coli* supplied resistance to wine toxicity, whereas no significant changes in *fts*H gene expression were found when *O. oeni* was subjected to ethanol stress. In *O. oeni* the synthesis of Lo18, an 18 kDa protein, is markedly induced under a variety of stress conditions and during stationary growth phase (17), representing a general stress marker in this bacterium. Moreover, Lo18 was found to be peripherally associated with the cytoplasmic membrane, and it was suggested that it could be involved in the maintenance of membrane integrity (20). This prompted us to investigate also the ethanol-induced association of proteins with the cytoplasmic membrane in *O. oeni* cells.

It is now well established that the survival of microorganisms in a variety of potentially lethal conditions can be improved by pre-exposure to sublethal stress conditions of the same kind. *O. oeni* cells

grown in the presence of 8% (vol/vol) of ethanol were able to maintain the efficiency of the cytoplasmic membrane as a semi-permeable barrier during ethanol challenge, by adjusting their membrane fluidity at the lipid-water interface (7). This could be partially explained by the membrane composition shift observed during growth in the presence of ethanol, i.e. a decrease in the total amount of lipids. This result hints at an important role for the protein content of membranes during ethanol adaptation. However, the effect of growth in the presence of ethanol on the integral-membrane proteins of *O. oeni* has never been established.

Proteomics is a very powerful tool to understand how organisms respond to environment stresses, though, the use of proteomic techniques in *O. oeni* is still limited. Radiolabeling with ^{35}S-methionine has been used to study protein profiles in this bacterium under stress conditions (16, 17). Immunoblotting has been used to study the expression of specific proteins, e.g. malolactic enzyme (14, 25), H^+-ATPase (45) and Lo18 (18, 20, 45).

In this paper we provide evidence for an active ethanol-protective response in *O. oeni*. Subsets of cytoplasmic, membrane-associated and integral membrane proteins were identified and their role in adaptation to ethanol is discussed.

5.3. Materials and Methods

Bacterial strain and growth conditions. *Oenococcus oeni* GM (Microlife Technics, Sarasota, Fl.) was cultured at 30°C in FT80 medium (pH 4.5) (4) modified by the omission of Tween 80, containing 10 g of DL-malic acid per litter. Glucose and fructose were autoclaved separately and added to the medium just before inoculation, at a final concentration of 2 and 8 g per litter, respectively. Early stationary phase cultures were diluted 100-fold in fresh medium, incubated for 24 h, and then used to obtain 1% inoculated cultures. In the adaptation experiments the culture medium was supplemented with 8 % (vol/vol) ethanol.

Stress conditions. Cells of *O. oeni* cultured in the absence and in the presence of 8 % (vol/vol) ethanol were harvested by centrifugation at exponential phase (20 h and 40h respectively), suspended in the same medium containing 12% (vol/vol) ethanol and incubated for one hour at 30°C. Cells were recovered by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and concentrated in the same buffer to an OD_{600} of 5.

Total protein extraction from *O. oeni*. Cells were disrupted by bead beating with an MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany) and zirconium beads (0.1-mm diameter; Biospec Products, Bartlesville, Okla.) six times for 1 min (with cooling on ice between treatments). Subsequently, proteins in the homogenate were analyzed by Western blotting and two-dimensional gel electrophoresis (2D-E). The protein concentration in cell extracts was determined by using the bicinchoninic acid assay (Sigma Chemical Co., St. Louis, Mo.).

Analysis of total protein by 2D-E. Total protein analysis was performed with a Multiphor 2D-E system (Pharmacia Biotech, Uppsala, Sweden) as described by Wouters et al. (49). Non-protein impurities in the sample (e.g. lipids) interfered with separation and subsequent visualization of the 2-D gels (data not shown), and therefore protein samples were subjected to acetone precipitation prior to loading (15). Equal amounts of protein (120 µg) were separated on isoelectric-point gels at pI 4 to pI 7 and subsequently on homogeneous sodium codicil sulfate-12 to 14% polyacrylamide gels (Pharmacia Biotech). The gels were stained with Coomassie brilliant blue. The experiments were performed in triplicate, and representative gels are shown. The gels were analyzed by using PD-Quest software (Bio-Rad, Richmond, Calif.) and standardized by calculating the intensity of each spot as the percentage of the total intensity of the spots visualized on a gel, after which the induction or repression factors were calculated.

Determination of N-terminal amino acid sequences of total proteins. For determination of the N-terminal amino acid sequences of specific spots, protein samples (1.5 mg) were separated on the 2D-E gels under conditions identical to those used for running of the analytical gels. The proteins were blotted on a polyvinylidene difluoride membrane optimized for protein transfer (Amersham Life Science, Buckinghamshire, England) with a Trans-Blot unit in accordance with the instructions of the manufacturer (Bio-Rad) and stained with Coomassie brilliant blue. Protein spots were cut from the blot and subjected to consecutive Edman degradation and subsequent analysis with the model 476A Protein Sequencing System (Applied Biosystems, Foster City, Calif.) at the Sequence Center, University Utrecht (Utrecht, The Netherlands). By using BlastP and the *O. oeni* PSU-1 genome sequence (sequenced by Doe Joint Genome Institute, University of California; www.jgi.doe.gov) deposited in the ERGO database (http://ergo.integratedgenomics.com/ERGO), the derived N termini were analyzed for sequence similarities.

Membrane protein extraction from *O. oeni*. Cells (3 g wet weight) were ressuspended in 12 ml of sample buffer (5 mM Mg-Acetate, 50 mM HEPES pH 7.5, 100 mM K-Acetate pH 7.5) disrupted by French pressing (3 times at 1000psi) and centrifuged (10.000xg, 10min, 4°C) to eliminate cell debris. After French-pressing all the steps were performed at 4°C and all the solutions and buffers added of minicomplete (protein inhibitor). The supernatant was recovered and centrifuged in a sucrose-gradient in order to recover cytoplasmic membranes. Supernatant was added of benzonase (125 U/l) and sucrose (0.5 M final concentration). Sucrose-gradient centrifugation was performed under conditions that stabilize ribosomes and consequently allowed ribosomal proteins to be removed easily: bottom layer: 3 ml of 2 M sucrose in sample buffer (5 mM Mg-Acetate, 50 mM HEPES pH 7.5, 100 mM K-Acetate pH 7.5, and 8 mM ß-mercaptoethanol); immediate up layer: 3 ml of 1,5 M sucrose in sample buffer; middle layer: 8 ml of supernatant (0,5 M sucrose) and the top layer: 2 ml of sample buffer without sucrose. After ultracentrifugation (27 000 rpm, rotor SW 28.1 Beckmann, 45min, 4°C) each membrane band was collected and diluted (1:1) in miliQ H_2O and ultracentrifuged for 1h at 27 000 rpm (rotor SW 28.1 Beckmann). In order to remove remaining cytoplasmatic proteins a series of washing steps were performed: membrane pellets were ressuspended in 5ml 1MTris (pH 7.5), incubated for 30min at 4°C and ultracentrifuged (50 000 rpm, rotor TLA 100.4 Beckman, 20min., 4°C). The pellets were ressuspended in

5ml 0,1M sodium carbonate (pH 11), ultra sonicated for 5min in a ice-bath, incubated for 30min on ice and ultracentrifuged (50 000 rpm, rotor TLA 100.4 Beckman, 20min., 4°C). The last procedure was repeated and the pellets were resuspended in 5 ml of miliQ H_2O to remove the sodium carbonate. The protein suspension was ultracentrifuged (50 000 rpm, rotor TLA 100.4 Beckman, 20min., 4°C) and the pellet ressuspended in 1 ml of solubilization buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM DTT, 0.5% w/v Pharmalyte pH 4-7, 1 mM Pefabloc SC, 2 µM Leupeptin). After ultracentrifugation at 50 000 rpm for 20min., the supernatant contains predominantly membrane associated proteins, whereas the pellet contains hydrophobic proteins which are not soluble in the solubilization buffer.

Membrane protein analysis. Membrane associated proteins of the supernatant were separated by two-dimensional gel electrophoresis essentially as described by O'Farrell (36). The isoelectric focusing (first dimension) was performed using the IPGphor system and Immobiline DryStrip (Amersham Biosciences) with linear immobilized pH gradients (pH 4-7, 18cm). The supernatants were diluted to a final volume of 360 µl with solubilization buffer and applied to the Immobiline gel strips by 12 h in-gel rehydration. The IEF was carried out sequential at 100V for 4h, 300V for 3h, 3000V for 6h, 5500V for 3h, and 5500V for 20h in a gradient mode at 20°C. For the second gel dimension the gel strips were incubated for two intervals of 15min in equilibration solution (6 M Urea, 30% glycerol, 2% w/v SDS in 0.05 M Tris/HCl buffer, pH 8.8), once added 1% w/v DTT to reduce the proteins and second added 4% w/v iodoacetamide to carbamidomethylated them. The strips were transferred to 12 to 15 % acrylamide gradient gels and the SDS-PAGE was performed overnight at 125 V and 10°C using the ISO-DALT electrophoresis system (Amersham Biosciences). The 2D-gels were stained with ruthenium II tris according to Rabilloud et al. (40). The separation of the hydrophobic proteins from the pellet was performed by 16 BAC-SDS-PAGE with a PROTEAN II system (Bio-Rad) as described by Hartinger et al. (19). The 16BAC-SDS-gels were stained with colloidal Coomassie brilliant blue G-250 according to Neuhoff et al. (35).

Mass spectroscopy analysis of membrane proteins. For mass spectrometry, proteins of the 2D-gels were handled as described by Kruft et al. (24). Proteins of the 16 BAC/SDS–gel were additionally carbamidomethylated before trypsin digestion. To reduce the cysteine residues proteins were incubated in 50µl of 20mM DTT/25mM NH_4HCO_3 for 30 min at 56°C. Excess liquid was removed and the gel pieces were incubated in 70µl acetonitrile. When the gel pieces had shrunk, the acetonitrile was discarded and 50µl of 55mM iodacetamide/25mM NH_4HCO_3 was added for 30 min in the dark. The gel pieces were dehydrated by acetonitrile and dried in a SpeedVak. Subsequently the gel pieces were incubated in 30µl of digestion solution containing 2ng/µl trypsin (Promega, Madison, WI). Mass measurements were performed by positive-ion MALDI/TOF-MS using a Bruker Reflex instrument equipped with delayed-extraction and a nitrogen laser (337nm). Zip-Tip elution was done with 1µl of matrix solution (19 mg α–cyano-4-hydroxycinnamic acid in 1ml of 60% methanol/0.1% formic acid), which was placed directly on the MALDI target. All spectra were recorded in the reflection mode with an acceleration voltage of 20 kV and a reflection voltage of 21.5 kV. Evaluation of the spectra was performed by using the BioTools package (Brüker Daltonik) and the identification was done using the Mascot search engine (Matrix science). Peptide

sequencing was performed on a quadruple time-of-flight mass spectrometry instrument (Q-tof II; Micromass) equipped with nanospray ion source. 3µl of Zip Tip-purified sample was filled into Au/ PD-coated nanospray glass capillaries (Protana), which was placed orthogonally in front of the entrance hole of the Q-tof. The generated sequences were compared to sequence entries in the *O. oeni* PSU-1 genome database (sequenced by Doe Joint Genome Institute, University of California; www.jgi.doe.gov). By using the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), a search for short, nearly exact matches was performed.

Additional Analytical Methods. Cellular dry weight was determined by filtering 80 ml of the cell suspensions through pre-weighed polyethylene filters, with a porosity of 0.22 µm, and dried at 100°C in an oven until a constant weight was reached. As a control the dry weight of the same volume of phosphate buffer was also determined. Protein was assayed by the method of Lowry et al. (29).

5.4. Results

2D-E of total protein of *O. oeni* cells. Cell free extracts from end-exponential cultures grown without and with 8% (vol/vol) of ethanol and from cultures shocked with 12% ethanol for 1 h were separated using 2D-E. Analysis of the 2D-EF gels revealed a total of approximately 100 proteins indicating low efficiency staining. Combined with the protein identification protocol, which involves coomassie staining after blotting, we decided to use coomassie staining throughout the research. The analysis of the gels showed that both ethanol-stress and adaptation changed the protein profiles of *O. oeni* cells (Fig. 5-1). The N-terminal sequences of a subset of proteins were determined and the respective function assigned from the annotation in the *O. oeni* genome sequence (Table 1). Stressing cells with 12% of ethanol for 1 hour promoted a decrease of several proteins, namely, spots 1, 3 and 4 (Fig. 5-1b, Table 2). These spots were identified as Metal-dependent hydrolase (MDH), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Inosine-5'-monophosphate dehydrogenase (IMPDH), respectively (Table 2). The synthesis of some proteins within the region of 14-22 kDa and with a pI 4.5-5.5 was also induced (Fig. 5-1b), including spot 5 (Table 2) identified as phosphocarrier protein HPr (HPr). The proteins corresponding to spots 8 and 10 were present only when cells were exposed to 12% of ethanol, suggesting that these proteins are typical ethanol-stress induced proteins. However, these two spots could not be identified since the signals were too low to allow a proper assignment of the N-terminal amino acid sequence.

Further analysis of 2D-EF gels revealed that a limited number of proteins were increased during growth in the presence of ethanol, mainly with high MW (spot 2, 12, Fig 5-1). Spot 2 was identified as glutathione reductase (GR) and was 3-fold induced (Fig. 5-1, Table 2). Notably, some spots were not detected in these ethanol-adapted cells including spot 1 and 5. Spot 5 corresponds to HPr, with a MW, predicted from the HPr amino acid sequence, of 9,1 kDa, however from the 2D-EF gel analysis we can infer a MW of approximately 17-18 kDa. N-terminal sequence analysis revealed two sequences that only differ in the Methionine of the first position (MVSKEFTI and XVSKEFTI). This suggests that HPr in *O. oeni* can be

present in a dimeric form. Dimerization of HPr and the HPr-like protein, Crh, has been reported previously in gram-positive bacteria (13, 21).

Spot	N-terminal sequence	MW (kDa)	Description
1	XELEFXXX MELEFLGT	36.2	Metal-dependent hydrolase
2	XGPQQYDY MKNQQYDY	48.6	Glutathione reductase
3	TVKIGINRFGRI TVKIGINGFGRI	37.1	Glyceraldehyde 3-P dehydrogenase
4	TDFSNKYT TDFSNKYT	40.1	Inosine-5'-monoP dehydrogenase
5	VSKEFTIT VSKEFTIT	9	Phosphocarrier protein HPr
6	AKGLVAGI AKGLVAGI	17.7	General stress protein[a]
7	c	-	-
8	XSELMXRX[b]	-	-
9	XLXXXKRV[b]	-	-
10	c	-	-
11	c	-	-
12	c	-	-

a = closest hit 37% similarity with *L. monocytogenes*
b = no hits found
c = signals were to low to give an assignment of a sequence

Table 5-1 List of cellular proteins of *O. oeni*. For each spot (numbering according to Fig. 5-1), the N-terminal sequence, the molecular weight (MW in kDa) and the possible function are indicated.

Finally, the protein corresponding to the spot 6 showed homology with a putative general stress protein in *L. monocytogens* (Table 1), however, no induction was observed during ethanol-shock or adaptation.

Integral membrane proteins of *O. oeni* cells. Integral membrane proteins contain one or more hydrophobic segments that are compatible with the hydrophobic interior of the lipid bilayer. The use of classical 2D-electrophoresis for the analysis of these proteins is problematic since many of these proteins resolve poorly in the dimension of the pH-gradient. This is partially due to the insolubility of membrane proteins in non-ionic detergents particularly at low ionic strength (despite the presence of a high concentration of urea). Moreover, because of their hydrophobicity these proteins tend to precipitate before reaching their isoelectric points (19). 16-BAC/SDS-PAGE provides a good tool for the characterization of membrane proteins (19). The first dimension is electrophoresis towards the cathode at acidic pH in the presence of the cationic detergent 16-BAC. Analysis of the gels revealed that ethanol stress and adaptation resulted in minor changes in the membrane protein composition (Fig. 5-2). Q-tof MS/MS analysis revealed that the mannose-specific IIAB component of the mannose PTS system (EII^{Man}) corresponding to spot 1 (Fig. 5-2C, table 3) was not present in ethanol grown cells. Spot 2, which features high homology to a Glutamine ABC transporter-ATP-binding protein, was decreased in ethanol-stressed cells. The protein corresponding to spot 3 was increased in ethanol-stressed cells. Q-tof analysis of this protein gave a good spectrum (EYVPALQEDLR), but no homologous sequences in the database of *O. oeni* were found. Even though several washing steps were done, we still found contamination with cytoplasmic proteins, since the analysis of spot 4 features high homology to phosphogluconate dehydrogenase (PGDH). The level of this protein was induced in ethanol-stressed cells whereas it was completely absent in ethanol-adapted cells.

Spot	Protein	Ethanol-stress	Ethanol-adaptation
1	MDH	0.6	nd
2	GR	1.1	3.6
3	GPDH	0.5	0.9
4	IMPDH	0.6	0.5
5	HPr	3	nd

nd = not detected

Table 5-2 Protein expression levels of *O. oeni* cells stressed with 12% of ethanol for 1 h and grown in the presence of 8% ethanol (numbering according to Fig. 5-1). Induction or repression factors were calculated as described in material and methods.

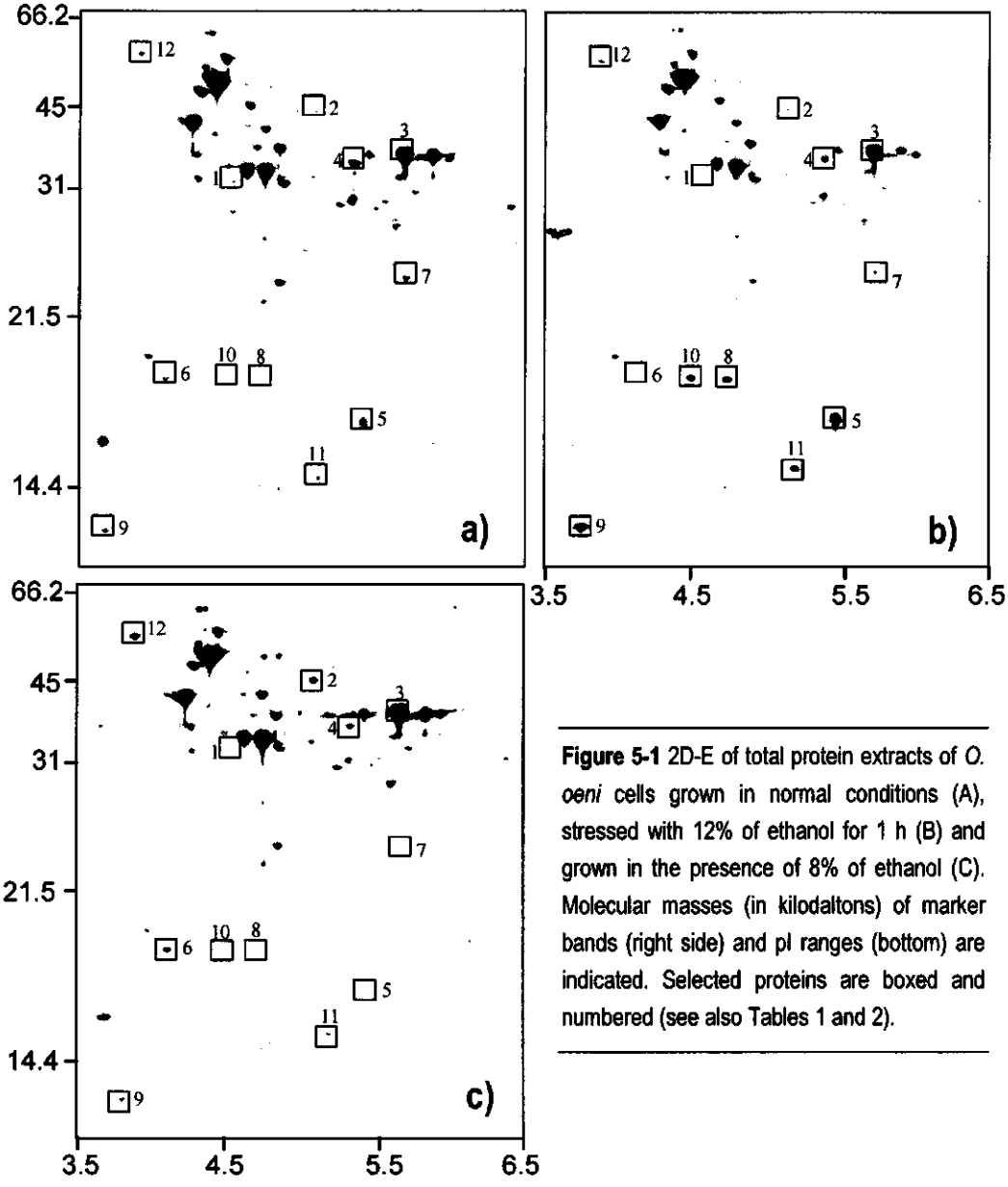

Figure 5-1 2D-E of total protein extracts of *O. oeni* cells grown in normal conditions (A), stressed with 12% of ethanol for 1 h (B) and grown in the presence of 8% of ethanol (C). Molecular masses (in kilodaltons) of marker bands (right side) and pI ranges (bottom) are indicated. Selected proteins are boxed and numbered (see also Tables 1 and 2).

Membrane associated proteins of *O. oeni* cells. Fractions with membrane associated proteins were isolated after sodium carbonate extraction. According to the prior treatment of the membranes these fractions contain proteins, which were attached to the membrane by protein-protein interaction, including the so-called lipoproteins, which are attached covalently to the membrane by acetylation. *O. oeni* cells grown in the presence of 8% (vol/vol) ethanol showed additional protein spots of putative membrane-

associated proteins than control cells (Fig. 5-3). From the 2-D-gels 12 protein spots were selected for analysis by mass spectroscopy and shown to include putative dTDT-glucose 4,6-dehydratase, D-alanine:D-alanine ligase, phosphomethylpyrimidine Kinase, cytidine monophosphate kinase and a protein belonging to the decarboxylase family (Tab. 4). All selected spots were only present in ethanol-grown cells (Fig. 5-3), except spot 1 that was absent in these cells.

Spot	Sequence of the peptide analyzed	MW (kDa)	Description
1	ALVLFENPEDALK	35.5	PTS system, mannose-specific IIAB (EII^{man})
2	AQQFVDQVQNH	27.2	Glutamine ABC transporter, ATP-binding protein
3	EYVPALQEDLR[b]	-	-
4	LIPSFTIEDFVK	53	phosphogluconate[a] dehydrogenase (PGDH)

a = cytoplasmic protein
b = no hits found

Table 5-3 Putative membrane proteins of *O. oeni*. For each spot (numbering according to Fig. 5-2), sequences of short single peptides, the molecular weight (MW in kDa) and the possible function are indicated.

5.5. Discussion

Performance of microorganisms under ethanol-stress conditions, as those prevailing in wine, requires specific cellular features, including modification of the protein composition to allow survival under such adverse conditions. We examined the proteome of *O. oeni*, a lactic acid bacterium of oenological interest, the genome of which has been sequenced recently by Miles (Doe Joint Genome Institute, University of California; www.jgi.doe.gov). Our main focus was the characterization of proteins involved in ethanol adaptation. To understand the physiological relevance of the site-specific location of these proteins, cytoplasmic, membrane-associated and integral-membrane proteins were investigated. Ethanol triggered alterations in protein patterns of *O. oeni* cells. From the 28 proteins analysed, 14 were identified as proteins with assigned function involved in a variety of cellular processes. The possible role of these proteins in ethanol adaptation is discussed.

Glutathione reductase (GR) catalyses the regeneration of glutathione (GSH) from glutathione disulphide (GSSG) at the expense of NADPH, and was more than 3-fold induced during ethanol adaptation.

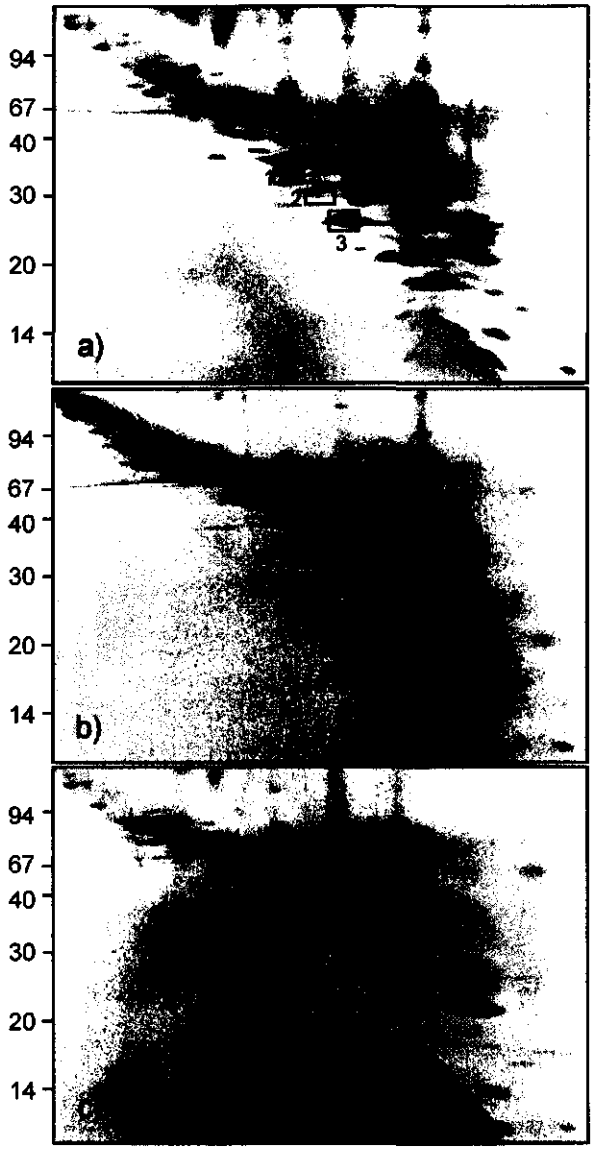

Figure 5-2 2D-separation of hydrophobic membrane proteins by 16-BAC/SDS-PAGE from *O. oeni* cells grown in normal conditions (A), stressed with 12% of ethanol for 1 h (B) and grown in the presence of 8% of ethanol (C). Molecular masses (in kilodaltons) of marker bands (right side and bottom) are indicated. Analyzed proteins are boxed and numbered (see also Table 3).

The first report of GR in Lactic acid bacteria appeared in 1995 by Pebay et al (37), providing new data concerning LAB responses to oxidative stress. Since the principal thiol-disulfide redox buffer in the cytoplasm of bacteria is thought to be GSH (48), GR is probably important for maintaining the redox potential at a low level for protection of thiol-containing proteins against oxidative stress. One of the known

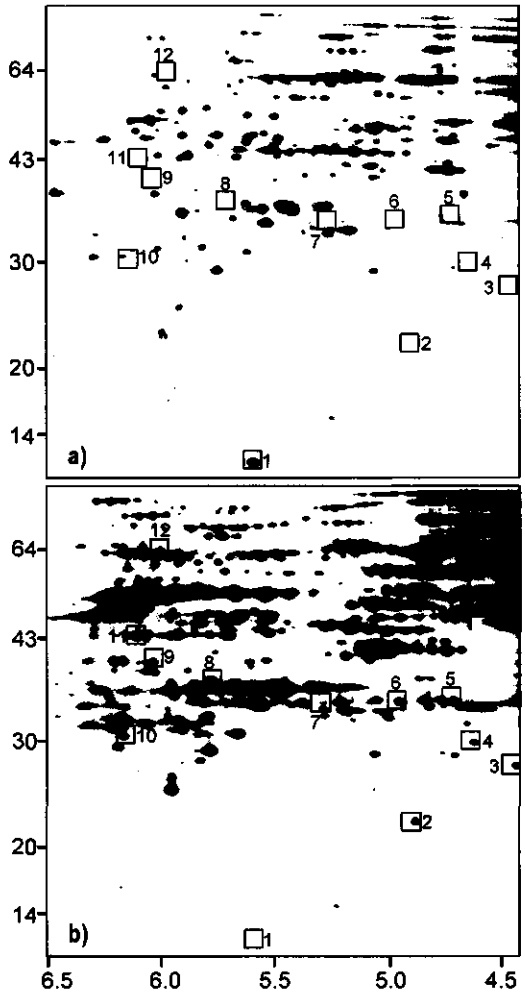

Figure **5-3** 2D-E of membrane-associated proteins of *O. oeni* cells grown in the absence (A) and in the presence of 8% of ethanol (B). Proteins were extracted from the same amount of cells. The same samples were used to extract integral membrane proteins (see above and material and methods). Molecular masses (in kilodaltons) of marker bands (right side) and pI ranges (bottom) are indicated. Selected proteins are boxed and numbered (see also Table 4)

toxic effects of acetaldehyde accumulation as a consequence of ethanol oxidation comes from the reaction of the aldehyde group with thiol compounds, such as cystein and glutathione (Rawat, 1975 and Lieberthal, 1979 in [22]). Moreover, it has been shown that ethanol increases dramatically the production of reactive oxygen species (ROS) in yeast, which cause severe oxidative damage to proteins, lipid and DNA (33). Thus, the increase of GR observed in *O. oeni* ethanol-adapted cells suggests that the cells have to cope with oxidative damage during ethanol adaptation.

Inosine monophosphate dehydrogenase (IMPDH) catalyzes the conversion of IMP to XMP with the concomitant reduction of NAD^+ to NADH. This reaction is the rate-limiting step in guanine nucleotide biosynthesis (6). We observed that the synthesis of IMPDH was inhibited in ethanol-adapted cells. Conceivably this inhibition will result in depletion of guanine nucleotides in these cells. The physiological effect remains to be elucidated.

Spot	MW (kDa)	Description	Closest Hits
3	22.1	Decarboxylase family protein	*E. faecalis* (62%)
7	29.2	Phosphomethylpirimidine kinase	*E. faecalis* (69%)
9	37.5	dTDP-glucose 4,6-dehydratase	*L. monocytogenes* (82%)
10	25.0	Cytidine monophosphate kinase	*L. monocytogenes* (74%)
11	42.1	D-alanine- D-alanine ligase	*Leuc. Mesenteroides* (70%)

Table 5-4 List of membrane-associated proteins of *O. oeni*. For each spot (numbering according to Fig. 5-3), the molecular weight (MW in kDa), the possible function and the closest hits are indicated.

In lactic acid bacteria the transport and phosphorylation of glucose is carried out by the phosphoenolpyruvate:mannose phosphotransferase system (PTS) (5). Phosphocarrier protein HPr (HPr), a general non-sugar-specific component of energy-coupling proteins and the sugar-specific EII complex (EII^{Man}) of the PTS (38), were not present in ethanol-adapted cells, suggesting that the mannose PTS is absent in these *O. oeni* cells. It is now well established that in *Bacillus subtilis* and other low-GC-content Gram-positive bacteria, the dominant catabolic repression (CR) pathway involves HPr and a transcription regulator, CcpA (for a review see [43]). HPr plays a role in CR as a corepressor of CcpA when phosphorylated at Ser-46 (11). It has also been shown, that mutations rendering the EII^{Man} complex inactive have a pleiotropic effect on an inducible fructose PTS activity (3) and results in an increased rate of PEP dependent phosphorylation of fructose (5). Thus, our results strongly suggest that adaptation to ethanol is associated with a relief of CR exerted by glucose over other carbon sources in *O. oeni* cells. This

represents a metabolic advantage to *O. oeni* during MLF since glucose is known to induce inhibition of malolactic enzyme activity via NADH accumulation during glucose metabolism (32). Moreover, phosphomethylpyrimidine Kinase (HMP-P kinase) which catalyses the stepwise phosphorylation of HMP-P into HMP-PP (23) a heterocyclic intermediate in the *de novo* synthesis of thiamine diphosphate (ThDP), was increased in ethanol adapted cells. The only ThDP-dependent enzyme known in non aerobic bacteria is xylulose 5-phosphate (X5P) phosphoketolase a central enzyme of the pentose phosphate pathway (PKP) of heterofermentative LAB (41, 47). Since the X5P concentration is expected to be low in ethanol-adapted cells (PGDH is almost absent) a possible explanation for the putative increased activity of X5P phosphoketolase in these cells is that pentoses e.g., xylose are taken up, converted into X5P and then fueled into the PKP pathway. However, this hypothesis presupposes that mechanisms involved in CR are offset (27) in ethanol-adapted *O. oeni* cells, as discussed above. These findings are in line with our observation that ATP synthesis and malic acid degradation are much more efficient in ethanol-adapted cells (8).

The presence of 8% of ethanol in the growth medium promoted a shutdown in the synthesis of several integral-membrane proteins. In contrast, additional protein spots which may be associated with the membrane were found in ethanol-grown cells compared to control cells, suggesting that cytoplasmic proteins can assume the function of stabilising the *O. oeni* cytoplasmic membrane in response to the ethanol-induced membrane disordering (7). It was previously described that the membrane association of Lo18, a general stress marker in *O. oeni* that is peripherally associated with the cytoplasmic membrane, was increased significantly upon temperature upshift (10). Torok et al. (44) presented evidence that the soluble chaperonin GroEL from *E. coli* can associate with model lipid membranes, and that binding was apparently governed by the composition of the host lipid bilayer, suggesting lipid-protein interactions. It is conceivable, that ethanol-induced hydrophobicity of cytoplasmic proteins (39) may increase the affinity of these proteins toward membranes, which may offer an explanation for the high level of membrane-associated proteins displayed in ethanol-adapted cells. Interestingly, two of these associated-proteins were assigned with functions that can be associated with the biosynthesis of cell wall: dTDT-glucose 4,6-dehydratase (4,6-dehydratase) that catalyses the irreversible conversion of dDTD-glucose to dTDT-4-keto-6-deoxyglucose (31) and D-alanine:D-alanine ligase (D-ala:D-ala ligase), which is an ATP-dependent enzyme that promotes dipeptide formation of D-Ala-D-Ala (1). The 6-deoxysugars are principle components of bacterial lipopolysaccharides (26) and in many prokaryotes D-Ala-D-Ala is incorporated into UDP-muramyl pentapeptide, which ultimately is used to produce peptidoglycan polymers (12).

Formerly, we reported that the protective effect of growth in the presence of ethanol is, to a large extent, based on modification of the physicochemical state of the membrane, i.e. *O. oeni* cells adjust their membrane permeability by decreasing fluidity at the lipid-water interface. Moreover, ethanol-adapted cells displayed increased metabolic capacity, i.e. revealed to be much more efficient in degrading malic acid and generating ATP than control cells (8). In this study, using a proteomic approach we provide evidence for an active ethanol adaptation response of *O. oeni* including the modification of cytoplasmic and membrane

protein profiles, reflecting the diversity of physiological responses. Three possible sites for cellular adaptation to ethanol are proposed: (i) Cell wall: dTDT-glucose 4,6-dehydratase and D-alanine:D-alanine ligase that were increased in ethanol-adapted cells are known to be involved in lipopolysaccharide and peptidoglycan biosynthesis, respectively, suggesting that cell wall is modulated during ethanol adaptation; (ii) Cytoplasmic membrane: the observed association between proteins and membranes during growth in the presence of ethanol may constitute a general mechanism that preserves membrane integrity during ethanol-stress; (iii) Metabolic pathways: Amounts of IMPDH and PGDH, that generate reduced nicotinamide nucleotides, are reduced during growth in the presence of ethanol, while GR that consumes NADPH is increased, suggesting that maintaining the redox balance plays an important role in ethanol-adaptation. These results are in agreement with previous observations that ethanol-adapted cells contain very low levels of NAD(P)H (8). Furthermore, two components of mannose PTS, i.e. HPr and EII^{Man}, were lacking in ethanol-adapted cells, providing strong evidence that mannose PTS is absent in ethanol-adapted cells, which represents a metabolic advantage to *O. oeni* cells during MLF. Finally, the increased level of HMP-P kinase involved in the *de novo* synthesis of ThDP, which is a coenzyme of X5P phosphoketolase a central enzyme of the PKP pathway, provides further evidence that ethanol-adapted cells are metabolically more active than control cells. Besides the above mentioned proteins that are involved in a variety of cellular processes, a putative general stress response protein and a protein involved in oxidative stress (GR) were also identified.

In this paper we present evidence that the physiological significance of ethanol adaptation in *O. oeni* cells is reflected in its proteome. In addition to the introductory global view of the *O. oeni* proteome when cells are grown in different environmental conditions, the various protocols described here provide new tools to achieve deeper insight in the protein composition and functionality of the *O. oeni* membrane.

5.6. Acknowledgments

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6. CONCLUDING REMARKS

6.1. Abstract

The control of activity of lactic acid bacteria (LAB) that carry out malolactic fermentation (MLF) is an important feature of the technology of modern commercial wine production. *Oenococcus oeni* is recognized as the principal microorganism responsible for the MLF under stress conditions as those prevailing in wine. MLF decreases the total acidity, improves the microbiological stability and contributes to the complexity of the "flavour" of wine. However, inoculation of *O. oeni* starter cultures directly into wine often results in failure of MLF. In this thesis we attempt to get insight into mechanisms involved in ethanol toxicity and acquired ethanol tolerance in this organism, which may supply tools to improve the practical application of reliable ready-to-use starter cultures of *O. oeni*. Although the cell damaging effects of ethanol are complex, strategies to obtain *O. oeni* starter cultures with enhanced ethanol tolerance are discussed.

6.2. Ethanol stress effect

The work described in this thesis clearly shows that ethanol acts as a disordering agent of the *O. oeni* cytoplasmic membrane, leading to leakage of intracellular material absorbing at 260 nm (chapter 2), promoting the passive influx of protons (chapter 2) and instantaneously increasing membrane fluidity mainly at the lipid-water interface (chapter 3). Typical compounds absorbing at 260 nm include NAD^+, NADH and AMP (17). NAD^+ is an important cofactor of the malolactic enzyme and it has been reported that the delicate balance between NAD^+/NADH affects the activity of the enzyme (21). Conceivably unspecific leakage of intracellular material significantly disturbs this balance, and consequently cell metabolism will be negatively affected. Moreover, ethanol-induced proton influx is expected to affect physiological processes in *O. oeni* that are dependent on a pH gradient, like ATP synthesis (chapter 4) and L-malate uptake (23).

Employing flow cytometry exposure to 12% (vol/vol) of ethanol was shown to result in a subpopulation (13% of the total population) that performs MLF i.e. these cells extrude carboxyfluorescein (cF) efficiently (Chapter 4). Inoculation with high cell densities of *O. oeni* has long been used for the induction of MLF in wine (12, 20). MLF only starts when the population of *O. oeni* reaches 10^6 CFU/ml in wine (18), however, under non-proliferating conditions, the minimal inoculation concentration to achieve optimal MLF is about 10^7 -10^8 CFU/ml (19). These results are in line with our observation that only approximately 13% of the population is metabolically active and consequently responsible for the MLF. Actually, inocula lower than 10^7 CFU/ml require a subsequent phase of cellular growth usually involving weeks or months and are not always successfully accomplished (19).

In *L. lactis* cells cF extrusion takes place via a primary transport system, which is most probably ATP dependent (2). During MLF ATP synthesis is dependent on the magnitude of the pmf (22). So, the inability of *O. oeni* to extrude the probe after ethanol stress is associated with the absence of pmf, and conceivably reflects the loss of the capacity to generate metabolic energy. Furthermore, when *O. oeni* cells were energized with glucose plus fructose instead of malic acid they were also negatively affected by ethanol in their ability to extrude cF (chapter 4), suggesting that other mechanisms than pmf dissipation are involved in inhibition of ethanol-induced cF-efflux including low pH_{in}. Moreover, it is known that the ratio NAD(P)H/ $NAD(P)^+$ plays an important regulatory role in multiple interlocked pathways in *O. oeni* and consequently in the global metabolism (21). Notably, addition of 12% (vol/vol) of ethanol to de-energized cells of *O. oeni* increased strongly the intracellular levels of NAD(P)H. Although NAD(P)H is unable to inhibit the malolactic activity of the pure enzyme, in whole cells a significant inhibitory effect is observed, which can be explained by the conversion of NADPH to NADH by transdehydrogenase activity (21). Several studies have reported the inhibition of the malolactic enzyme by NADH (1) that competes efficiently for the binding site of NAD^+, thereby causing inhibition.

6.3. Performance after ethanol adaptation

T he effect of pre-adaptation to ethanol was studied by subjecting *O. oeni* cells grown in the presence of 8% (vol/vol) to challenge concentrations of this compound. Although these cells were permeabilized to a certain extent by ethanol, ethanol-adapted cells showed lower passive proton influx rates at ethanol concentrations above 10% (vol/vol), especially at low pH (chapter 2). Thus, physiological processes in *O. oeni*, which are dependent on a pH gradient, like ATP synthesis (chapter 4) and L-malate uptake (23) are expected to be less affected in ethanol-adapted cells. Moreover, these cells were also able to retain more

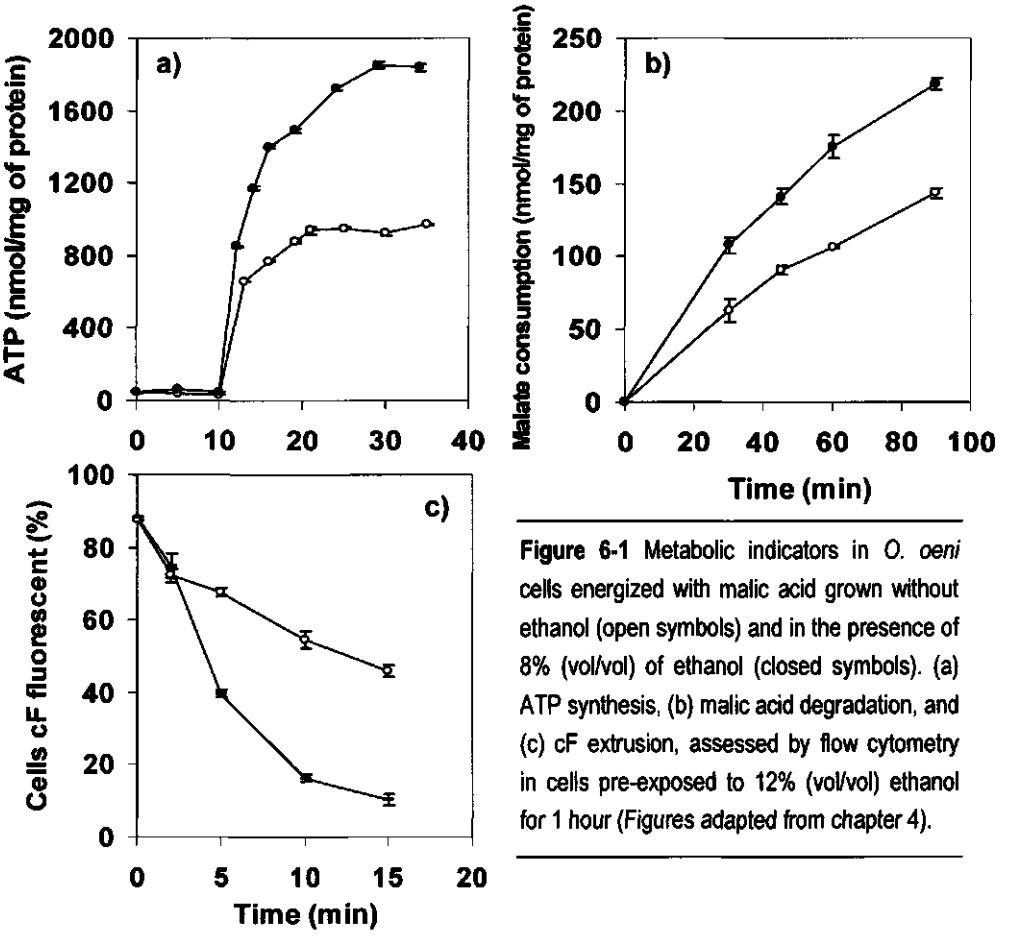

Figure 6-1 Metabolic indicators in *O. oeni* cells energized with malic acid grown without ethanol (open symbols) and in the presence of 8% (vol/vol) of ethanol (closed symbols). (a) ATP synthesis, (b) malic acid degradation, and (c) cF extrusion, assessed by flow cytometry in cells pre-exposed to 12% (vol/vol) ethanol for 1 hour (Figures adapted from chapter 4).

efficiently intracellular compounds including compounds absorbing at 260 nm (Chapter 2), and possibly manganese, an important cofactor of malolactic enzyme. Indeed, high levels of intracellular ATP (Fig. 6-1a) and higher rates of malic acid degradation (Fig. 6-1b) were observed in ethanol-adapted cells. The positive effect of ethanol adaptation on MLF is outlined in the Fig. 6-1c: growing cells in the presence of 8% (vol/vol)

ethanol appeared to suppress the ethanol-induced inhibition of cF extrusion, given that after being exposed to 12% (vol/vol) of ethanol for 1 hour these ethanol-adapted cells extruded cF very efficiently (Fig. 6-1c).

6.4. Mechanisms involved in ethanol adaptation

Using a proteomic approach we provide evidence for an active ethanol adaptation response of *O. oeni* at different cellular locations including cytoplasmic, membrane-associated and integral membrane proteins, reflecting the diversity of physiological responses mentioned above. Enzymes that generate reduced nicotinamide nucleotides are strongly inhibited during growth in the presence of ethanol, i.e. inosine-5'-monophosphate dehydrogenase (IMPDH) and phosphogluconate dehydrogenase (PGDH), while glutathione reductase (GR) that consumes NADPH was induced (chapter 5) suggesting that maintaining the redox balance plays an important role in ethanol-adaptation. These results are in agreement with the observation that ethanol-adapted cells contain very low levels of NAD(P)H (chapter 4). Furthermore, two components the phosphoenolpyruvate:mannose phosphotransferase system (PTS), i.e. Phosphocarrier protein HPr (HPr) and the sugar-specific EII complex (EII^{Man}), are lacking in ethanol-adapted cells, providing strong evidence that mannose PTS is absent in ethanol-adapted cells. In LAB the transport and phosphorylation of glucose is carried out by the mannose PTS (16, 29). Thus, our results suggest that transport of glucose is negatively affected in ethanol-adapted cells, which represents a metabolic advantage during MLF since glucose metabolism induces inhibition of malolactic enzyme activity due to NADH accumulation (21).

Although, there is a universal believe that changes in membrane permeability are associated with changes in the lipid order, a correlation between these two parameters was never established. Fig. 6-2 shows a strong negative correlation (r=-0.93) between the order parameter (S) values and cF-leakage-rates, which allows us to conclude that *O. oeni* cells adjust their membrane permeability during growth in presence of 8% (vol/vol) of ethanol by decreasing fluidity at the lipid-water interface. Interestingly, the addition of 8% (vol/vol) ethanol to these ethanol-adapted cells resulted in a membrane fluidity (at the position of the nitroxide label of 5-DS) that was similar to that in non-adapted cells in the absence of ethanol (chapter 3). This result implies that ethanol-induced adaptation of membrane fluidity is not only qualitatively, but also quantitatively consistent with the homeoviscous theory validated for bacteria by Sinensky (25).

A significant increase in the degree of unsaturation of the fatty acids was found in cells grown in the presence of 8% (vol/vol) ethanol which in terms would lead to an increased fluidity. However, as determined by ESR, the cytoplasmic membranes of these cells are much more rigid than those of control cells. The same phenomenon was observed in *E. coli* cells, cytoplasmic membranes of cells grown in the presence of 4% (vol/vol) of ethanol were more rigid than that of control cells, however, liposomes made from the phospholipids of these ethanol-grown cells displayed, as expected, increased fluidity (7). This observation could point to an important role for the protein content of membranes in regulating fluidity. The

presence of 8% of ethanol in the growth medium promoted a decrease of several integral-membrane proteins, whereas, an increased number of membrane-associated proteins was observed (chapter 5). The

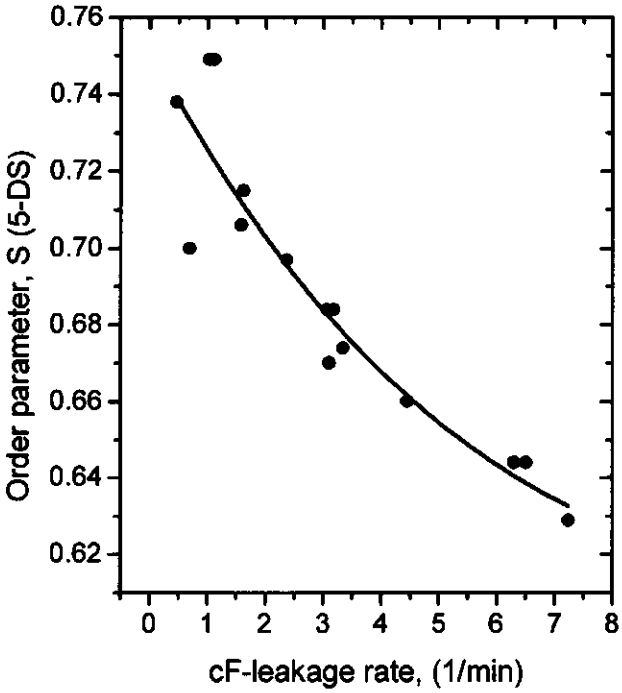

Figure 6-2 Correlation between order parameter (S) values and cF-leakage-rate. The data relate to *O. oeni* cells grown without (control) or with 8% ethanol and cells pre-exposed for 2h to 12% ethanol in the presence or absence of CAP. The cells were exposed to 0, 8, 12 and 16% ethanol during ESR spectra recording and cF-efflux measurements.

increased protein levels may offer an explanation for the higher rigidity displayed during growth in the presence of 8% (vol/vol) ethanol (chapter 3). Torok et al. (26) found that the GroEL chaperonin can associate with model lipid membranes and that binding was governed by the composition and the physical state of the lipid bilayer. GroEL was shown to increase the lipid order in the liquid crystalline state, yet remained functional as a protein-folding chaperonin. This suggests that chaperonins can assist the folding of both soluble and membrane-associated proteins while concomitantly stabilizing lipid membranes. Moreover, the rigidifying effect observed during ethanol-adaptation was most evident at the membrane lipid-water interface (chapter 3), where stress proteins are supposed to interact (27). It was previously described that the membrane association of Lo18, a general stress marker in *O. oeni* that is peripherally associated with the cytoplasmic membrane, was increased significantly upon temperature upshift, which may constitute part of a general mechanism that preserves membrane integrity during stress.

Several examples have been reported in which a clear correlation between membrane protein activity and the physical state of the membrane lipids was found (5, 13, 14, 28). The effect of membrane fluidity on H^+-coupled leucine transport was demonstrated in *Lactococcus lactis* in which the membrane fluidity was varied by the introduction of monolayer lipids, extracted from an extreme thermophilic archaea *Sulfolobus acidocaldarius* (11). The dynamics of the lactose permease of *E. coil* was found to be affected

by the physical state of dimyristoyl-phosphadityicholine (DMPC) (8), such that the transport rate drastically decreased below the lipid-phase transition, while binding of lactose was unaltered. *O. oeni* cells pre-exposed to 12% (vol/vol) ethanol for 2 hours were more rigid (more resistant to ethanol-induced membrane disordering) than control cells, although the degree of unsaturation and the total amount of lipids of these pre-exposed cells were identical to control cells (chapter 3). These results suggest that the physical state of the membrane plays an important role during ethanol-adaptation in *O. oeni*, possibly by controlling membrane associated processes, e.g. ATPase activity and other transport systems.

6.5. Contribution to wine industry

Although flow cytometry is a promising tool, its application to monitor fermentation processes is still limited. Given the speed of analysis and the diversity of cellular characteristics that can be measured, flow cytometry could be applied for online-monitoring providing detailed information about fermentation processes. Thus, it represents a sophisticated tool that may allow direct regulation of the process conditions in order to optimize the production. Therefore, it was our approach to evaluate the validity of the fluorescence assays as indicators for membrane integrity and metabolic activity in *O. oeni*.

We reported evidence that flow cytometry is a powerful technique to monitor in real time and with a high degree of statistical resolution, the physiological state of individual cells during malolactic fermentation. This information is of major importance in the study of MLF in wine, in order to assign bulk activities measured by classical methods, which assume that all the cells will contribute equally to the global performance of MLF, to the very active cells that are effectively responsible for the observations. We provide a rapid method to assess membrane integrity of *O. oeni* cells (Chapter 2), of major interest to the starter culture industries as an indicator of the physiological state of the individual *O. oeni* cells and to assess the bioenergetics of individual *O. oeni* cells (chapter 4), which represents a powerful technique to wine industry allowing online monitoring of malolactic activity at the single cell level.

Finally, the fundamental research underlying the elucidation of ethanol-induced inhibition of malolactic fermentation and the mechanisms involved in ethanol-adaptation accomplished in this work may provide useful directives for improvement of starter cultures focused on high activity performance under ethanol (and more generally wine) stress conditions.

6.6. Future work

Despite the understanding of mechanisms involved in the regulation of stress responses at the DNA level, little is known about how stress is detected by cells. Several experiments illustrate the link and cross-talk between the membrane composition and gene regulation (4, 5, 6, 10). In this thesis we demonstrate that ethanol-adapted cells modulate the composition of their membrane by increasing the percentage of unsaturated fatty acids and decreasing the total amount of lipids. In order to investigate whether modification of membrane composition affects *de novo* protein synthesis in *O. oeni,* cells were grown in

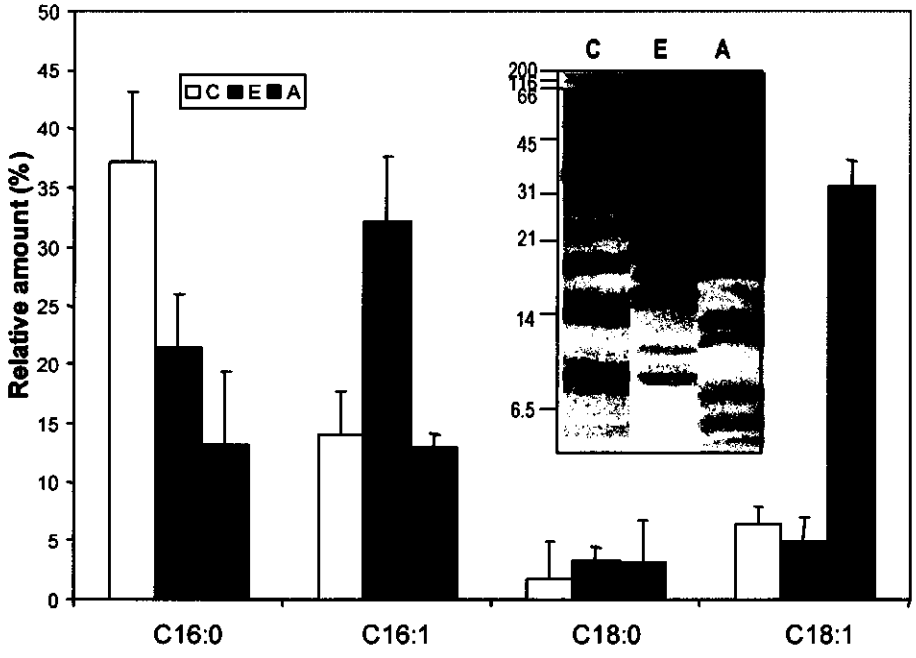

Figure 6-3 Percentage of individual fatty acids in *O. oeni* cells; control cells (C), cells grown in the presence of 8% (vol/vol) ethanol (E), and cells grown in lipid depleted medium in the presence of avidin and oleic acid (A), cells were recovered at the end of exponential phase (24h, 48h and 24h respectively). Inset, SDS-PAGE of 35S-methionine labeled proteins of the respective cells. The cells were washed and ressuspended in MAM20 to a final concentration of 0.5 mg protein/ml After 10 minutes of incubation labeling was carried out with 10 μCi/ml of L-[^{35}S] methionine for one hour. Cells were recovered by centrifugation and the cell pellets were washed with 10 mM Tris HCl, 1 mM EDTA, pH 8. The cell pellets were ressuspended in 120 μl of PMSF, and cells were disrupted by bead beating. Subsequently, 15 μl of proteins homogenate of each sample was loaded on the SDS-15%PAGE gel.

lipid depleted medium in the presence of avidin, which strongly and specifically inhibits *de novo* synthesis of fatty acid (24) and with oleic acid supplied as an external source of unsaturated FA. This provided a good system to study the influence of fatty acids on the *O. oeni* membrane *in vivo*.

O. oeni cells grown in the presence of ethanol contained less C16:0 than control cells, most likely as a result from the preferential inhibition of saturated fatty acid biosynthesis as observed in *E. coli* (3, 9), and an increase in the amount of C16:1 was observed (Fig. 6-3). Thus, the unsaturated FA/saturated FA ratio was much higher in ethanol-adapted cells than in control cells i.e. 1.5 and 0.5, respectively. Membranes from avidin-grown cells contained also less C16:0 than control cells, but they increased the unsaturation level of their membranes by incorporating the supplied oleic acid (C18:1) (Fig. 6-3). Although the protein profile of avidin-grown cells is not identical to ethanol-adapted cells, the results show that modification of the membrane composition affects the *de novo* protein synthesis in *O. oeni* cells (Fig 6-3 inset).

The approach described above may provide a tool to analyze the influence of membrane composition on stress signaling-pathways in *O. oeni*. Furthermore, the fact that ethanol-adapted cells modulate the composition of their membrane and that membrane composition itself affects protein synthesis, offers the perspective that it may be possible to engineer ethanol resistance into *O. oeni* cells by modifying the membrane composition. The recent completion of the *O. oeni* genomic sequence (Doe Joint Genome Institute, University of California; www.jgi.doe.gov) together with emerging proteomic techniques, such as isotope-code affinity tags (ICATs) and tandem mass spectrometry (15), stand to provide means to make this feasible.

6.7. References

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SUMMARY

The control of activity of lactic acid bacteria (LAB) that carry out malolactic fermentation (MLF) is an important feature of the technology of modern commercial wine production. The practical application of commercial malolactic starter cultures of *Oenococcus oeni* surviving direct inoculation in wine requires insight into mechanisms involved in ethanol toxicity and tolerance in this organism. Starter cultures should be seen as "reservoirs" that maintain malolactic enzyme in optimal conditions to perform MLF. Because of the optimum pH of the enzyme (around 5.8), the need for cofactors (Mn^{2+}, NAD^{+}), and the inhibitory effects of many wine components (carboxylic acids, polyphenols), the protein must be protected from the medium by the cell membrane. Thus, the malolactic activity of the cells is strictly dependent on the integrity of the plasma membrane.

Exposure to ethanol resulted in an increase of the permeability of the cytoplasmic membrane enhancing passive proton influx and concomitant loss of intracellular material (absorbing at 260 nm) (chapter 2). Cells grown in the presence of 8% (vol/vol) ethanol revealed adaptation to ethanol stress, since these cells showed higher retention of compounds absorbing at 260 nm. Moreover, for concentrations higher than 10% (vol/vol) lower rates of passive proton influx were observed in these ethanol-adapted cells, especially at pH 3.5. The effect of ethanol on *O. oeni* cells was studied using the ability to retain efficiently carboxyfluorescein (cF) as an indicator of membrane integrity and enzyme activity, and the uptake of propidium iodide (PI) to assess membrane damage (chapter 2). Flow cytometric (FCM) analysis of both ethanol-adapted and non-adapted cells with a mixture of the two fluorescent dyes cF and PI, revealed three main sub-populations of cells i.e. cF-stained, intact cells; cF and PI-stained, permeable cells; and PI-stained, damaged cells. The sub-population of *O. oeni* cells that maintained their membrane integrity, i.e. cells stained only with cF, was 3 times larger in the population grown in presence of ethanol, reflecting the protective effect of ethanol adaptation. Moreover, active extrusion of cF was used to assess malolactic activity in *O. oeni* (chapter 4). Upon the addition of malate to control cells cF efflux was strongly induced, i.e. after 5 min almost all the cells had lost the cF (95%). Flow cytometric analysis of cells pre-exposed to 12% (vol/vol) of ethanol for 1h, showed that two sub-populations could be distinguished, one of which being able to extrude cF during MLF (13%), i.e. cells non-fluorescent and the other one that lost the ability to extrude actively the probe, i.e. cells cF-fluorescent. This information is of major importance in the study of MLF in wine, in order to assign bulk activities measured by classical methods, which assume that all the cells will contribute equally to the global performance of MLF, to the very active cells that are effectively responsible for the observations.

Addition of 12% (vol/vol) of ethanol promoted a decrease in the ATP pool in cells performing MLF and a rapid increase in the level of intracellular NAD(P)H in deenergized cells. The last phenomenon was also observed upon the addition of a concentration of ethanol in the physiological range (100 mM), suggesting that a NAD(P)H/NAD(P)H unbalance is generated as a consequence of ethanol oxidation (chapter 4). Notably, cells grown in the presence of 8% (vol/vol) of ethanol were able to extrude cF during MLF after exposure to 12% (vol/vol) of ethanol for 1h. These cells displayed higher ATP and lower NAD(P)H pools under ethanol-stress conditions. The data give a coherent biochemical basis to understand the ethanol-induced inhibition of MLF in *O. oeni*.

The effect of ethanol on the cytoplasmic membrane of *Oenococcus oeni* cells and the role of membrane changes in the acquired tolerance to ethanol were investigated (chapter 3). Membrane tolerance to ethanol was defined as the resistance to ethanol-induced cF leakage. To probe fluidity of the cytoplasmic membrane, intact cells were labeled with doxyl-stearic acids and analyzed by electron spin resonance spectroscopy. Although the fluidizing effect of ethanol was noticeable across the width of the membrane changes at the lipid-water interface were more evident. Cells responded to growth in the presence of 8% (vol/vol) of ethanol by increasing membrane rigidity at the lipid-water interface. Upon exposure to a range of ethanol concentrations, these adapted cells had reduced fluidity as compared with cells grown in the absence of ethanol. Interestingly, the addition of 8% (vol/vol) of ethanol to cells grown in presence of 8% (vol/vol) of ethanol resulted in a membrane fluidity that was similar to that in non-adapted cells in the absence of ethanol. This result implies that ethanol-induced adaptation in membrane fluidity is not only qualitatively, but also quantitatively consistent with the homeoviscous theory.The degree to which the effects of ethanol on membrane permeability and fluidity share common features was investigated and a strong correlation between fluidity and cF-leakage was found for all treatments and alcohol concentrations tested. Thus, suggesting that the protective effect of growth in the presence of ethanol is, to a large extent, based on modification of the physicochemical state of the membrane, i.e. cells adjust their membrane permeability by decreasing fluidity at the lipid-water interface. Analysis of the membrane composition revealed an increase in the degree of fatty acid unsaturation and a decrease in the total amount of lipids in the cells grown in the presence of 8% (vol/vol) of ethanol. This observation would hint at an important role for the protein content of membranes in regulating fluidity. Actually, we found that cells grown in presence of ethanol displayed an increased number of membrane-associated proteins (chapter 5).

The site-specific location of proteins involved in ethanol adaptation including cytoplasmic, membrane-associated and integral-membrane proteins was investigated. We provide evidence for an active ethanol adaptation response of *O. oeni* including the modification of cytoplasmic and membrane protein profiles, reflecting the diversity of physiological responses. Levels of inosine-5'-monophosphate dehydrogenase (IMDH) and phosphogluconate dehydrogenase (PGDH) that generate reduced nicotinamide nucleotides, were decreased during growth in the presence of ethanol, while glutathione reductase (GR) that consumes NADPH, was induced suggesting that maintaining of the redox balance plays an important role in ethanol-adaptation. PTS components of mannose PTS including phosphocarrier

protein HPr (HPr) and the EII^{Man}, were lacking in ethanol-adapted cells providing strong evidence that mannose PTS is absent in ethanol-adapted cells, which represents a metabolic advantage to *O. oeni* cells during malolactic fermentation. Interestingly, dTDT-glucose 4,6 dehydratase and D-alanine:D-alanine ligase, that were increased in ethanol-adapted cells are known to be involved in lipopolysaccharide and peptidoglycan biosynthesis, respectively, suggesting that cell wall is also modulated during ethanol adaptation.

The fundamental research underlying the elucidation of ethanol-induced inhibition of malolactic fermentation and the mechanisms involved in ethanol-adaptation accomplished in this work may provide useful directives for improvement of starter cultures focused on high activity performance under ethanol (and more generally wine) stress conditions. Moreover, we provide a rapid method to assess membrane integrity of *O. oeni* cells (Chapter 2), of major interest to the starter culture industries as an indicator of the physiological state of the individual *O. oeni* cells and to assess the bioenergetics of individual *O. oeni* cells (chapter 4), which represents a powerful technique to wine industry allowing online monitoring of malolactic activity at the single cell level.

SAMENVATTING

Een belangrijk onderdeel van de commerciële wijnproductie is de zogenaamde malolactische fermentatie waarbij malaat (appelzuur) wordt omgezet in lactaat (melkzuur). Deze omzetting die wordt uitgevoerd door de melkzuurbacterie *Oenococcus oeni* is sterk bepalend voor de kwaliteit van de wijn. Voor een optimale beheersing van deze fermentatiestap zou tijdens de fermentatie een *O. oeni* starter kunnen worden toegevoegd. Door de ongunstige omstandigheden in de wijn waaronder de lage pH (pH 3.5) en de hoge concentratie ethanol (alcohol) wordt echter een groot aantal van de *O. oeni* cellen gedood. Het is daarom van groot belang om inzicht te verkrijgen in de ethanolgevoeligheid van *O. oeni* en in de capaciteit van dit organisme om resistentie te ontwikkelen tegen deze stress factor. Bij de malolactische fermentatie zijn twee enzymen betrokken: een malaat/lactaat transporteiwit dat zorgt voor opname van malaat, welke gekoppeld is aan de uitscheiding van lactaat, en het malolactisch enzym dat de omzetting katalyseert van malaat in lactaat. Dit laatste enzym werkt optimaal bij pH 5.8 en is afhankelijk van co-factoren (Mn^{2+} en NAD^{+}). Hiertoe dient dit intracellulaire enzym te worden afgeschermd van het extracellulaire milieu, de wijn, om optimaal te kunnen functioneren. Hierbij speelt de cytoplasmatische membraan een cruciale rol.

Blootstelling van *O. oeni* cellen aan ethanol resulteert in een verhoogde permeabiliteit van de cytoplasmatische membraan voor protonen en laag-moleculair cellulair materiaal (Hoofdstuk 2). Echter, wanneer *O. oeni* wordt voorgekweekt in aanwezigheid van ethanol [8% (vol/vol)] zijn de cellen veel resistenter tegen ethanol, hetgeen duidt op aanpassing van de cellen aan deze stress conditie. Bij blootstelling van deze cellen aan ethanolconcentraties boven de 10% (vol/vol) bleek de permeabiliteit voor protonen sterk te zijn verlaagd. Het effect van ethanol op de membraan permeabiliteit werd in detail onderzocht door de ethanol veroorzaakte uitstroom van de groen-fluorescerende verbinding, carboxyfluoresceïne (cF), die in de cel wordt gevormd door de enzymatische omzetting van de precursor, carboxyfluoresceïne-di-acetaat (cFDA), te analyseren in combinatie met een DNA kleuring door de rood-fluorescerende verbinding propidium-iodide (PI). Cellen met een intacte membraan worden groen gekleurd, terwijl cellen met een beschadigde membraan rood worden gekleurd. Met behulp van een flow cytometer werden vervolgens de individuele cellen geanalyseerd. Hieruit bleek dat blootstelling aan ethanol van *O. oeni* cellen gekweekt in afwezigheid en in aanwezigheid van 8% (vol/vol) ethanol resulteerde in drie typen cellen, namelijk groen (cF) gekleurde intacte cellen, groen-rood (cF-PI) gekleurde cellen met een permeabele membraan, en rood (PI) gekleurde beschadigde cellen. Het aantal intacte cellen was beduidend hoger indien de cellen waren voorgekweekt in aanwezigheid van ethanol, hetgeen duidt op aanpassing aan deze stress conditie. Daarnaast werd de energie-afhankelijke, transporteiwit-gemedieerde

uitscheiding van cF gebruikt als een indicator van de activiteit van de malolactische fermentatie (Hoofdstuk 4). Toevoeging van malaat aan controle cellen resulteerde in een snelle uitscheiding van cF. Flow cytometrische analyse van cellen blootgesteld aan 12% (vol/vol) ethanol, liet twee typen cellen zien, cF-gekleurde beschadigde cellen, en intacte niet-gekleurde cellen, welke cF hebben uitgescheiden. Een dergelijke aanpak biedt belangrijke mogelijkheden voor de selectie en analyse van die cellen welke nog steeds zeer actief zijn na blootstelling aan hoge concentraties ethanol.

Toevoeging van 12% (vol/vol) ethanol aan *O. oeni* resulteerde in een verlaging van de intracellulaire ATP-concentratie en een verhoging van de NAD(P)H concentratie. Cellen voorgekweekt in aanwezigheid van 8% (vol/vol) ethanol bleken in staat cF actief uit scheiden in aanwezigheid van 12% (vol/vol) ethanol. Kenmerkend voor deze cellen zijn de verhoogde malolactische fermentatie activiteit, de hoge intracellulaire ATP concentratie en de lage NAD(P)H concentratie.

Gezien de belangrijke rol van de cytoplasmatische membraan in de tolerantie voor ethanol is uitgebreid onderzocht wat voor effect ethanol heeft op de membraan van *O. oeni* en op wat voor manier de membraaneigenschappen worden aangepast tijdens blootstelling aan ethanol (Hoofdstuk 3). Het effect van ethanol op de vloeibaarheid van de membraan van *O. oeni* cellen werd onderzocht met behulp van spectroscopische technieken waarbij de beweeglijkheid van specifieke membraanoplosbare verbindingen werd geanalyseerd. Hieruit bleek dat ethanol vooral de vloeibaarheid aan het oppervlak van de membraan beïnvloedt dat wil zeggen op het membraanlipide-water grensvlak. In cellen gekweekt in de aanwezigheid van 8% (vol/vol) ethanol, bleek de membraanvloeibaarheid significant lager te zijn. Toevoeging van ethanol (8% vol/vol) resulteerde in een verhoging van de membraanvloeibaarheid vergelijkbaar met die in controle cellen in de afwezigheid van ethanol. Er bleek een duidelijke correlatie te zijn tussen de membraanvloeibaarheid en de ethanol-geïnduceerde cF uitlek. Dit suggereert dat in *O. oeni* de membraaneigenschappen worden aangepast tijdens groei in aanwezigheid van ethanol (8% vol/vol). Analyse van de vetzuursamenstelling van de membraanfosfolipiden liet een toename zien van het percentage zogenaamde onverzadigde vetzuren en een afname van de totale hoeveelheid lipiden in *O. oeni* cellen gekweekt in aanwezigheid van 8% (vol/vol) ethanol. Deze afname wordt mogelijk gecompenseerd door een toename van het percentage eiwitten in de membraan. Met name de waargenomen toename van de hoeveelheid membraan (geassocieerde) eiwitten (Hoofdstuk 5) zou een rol kunnen spelen bij het bepalen van de membraanvloeibaarheid.

Het effect van ethanol op cytoplasmatische en membraan(geassocieerde) eiwitten is verder onderzocht met behulp van twee-dimensionale gelelectroforese (Hoofdstuk 5). Hieruit komt duidelijk naar voren dat *O. oeni* de eiwit samenstelling op verschillende locaties in de cel kan aanpassen tijdens blootstelling aan ethanol waarbij sommige eiwitten verdwijnen en andere juist worden gevormd. Verschillende eiwitten konden na extractie uit de gel worden geïdentificeerd waaronder enzymen betrokken bij de handhaving van de redoxbalans in de cel (oa glutathion-reductase) en enzymen betrokken bij de celwandsynthese (o.a. D-alanine-D-alanine ligase). Zeer opvallend was het nagenoeg ontbreken van een

aantal enzymen dat specifiek is betrokken bij het suikermetabolisme o.a. enzym-II van het mannose fosfotransferase systeem (PTS), hetgeen de activiteit van de malolactische fermentatie ten goede komt, aangezien deze wordt geremd door suikers, met name glucose.

Dit onderzoek heeft enerzijds mechanistisch inzicht verschaft in de effecten van ethanol op de cytoplasmatische membraan en het metabolisme, met name de malolactische fermentatie, van *O. oeni*, en anderzijds is inzicht verkregen in de mechanismen welke ten grondslag liggen aan de adaptatie van dit organisme aan hoge concentraties ethanol. Deze informatie kan worden gebruikt bij de verdere optimalisatie en selectie van *O. oeni* starter cultures voor directe toevoeging aan wijn. Hierbij kunnen de in hoofdstuk 2 en 4 beschreven snelle methoden voor de bepaling van de membraan-permeabiliteit en de energetische toestand van individuele cellen in een populatie de industrie de mogelijkheid bieden om de malolactische activiteit van individuele *O. oeni* cellen te volgen tijdens de wijnproductie.

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Maria Graça

CURRICULUM VITAE

Maria Graça Amaral da Silveira born on December 30th, 1968, in Horta, Açores, Portugal, completed her secondary high school in 1986. She graduated in 1991 as an agro-industrial engineer by the Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal, with the final mark of 15 (over 20). In 1995 she became master in Food Science and Technology by Universidade Técnica de Lisboa, with the final mark of 17 (over 20).

During 1995 she collaborated on a STRIDE program/A: "Malolactic fermentation in wine metabolism, bioenergetic and regulation". A JNICT project STRDA/C/BIO/355/92 at Centro de Tecnologia Química e Biológica (CTQB), Laboratory of Microbiology, Oeiras, Portugal, supervised by Prof. Helena Santos. In 1996 she participated on a PIDDAC program: "Adaptation mechanisms of lactic acid bacteria in wine. Role of ethanol and other metabolites in malolactic fermentation", at Centro de Tecnologia Química e Biológica (CTQB), Laboratory of Microbiology, Oeiras, Portugal, supervised by Doctor M. V. San Romão.

In September 1998, she received a scholarship from the Fundação para a Ciência e Tecnologia, Portugal, to start her PhD program at the Laboratory of Food Microbiology, Wageningen University, on "Effects of ethanol on *Oenococcus oeni*: Stress response, adaptation and performance".

After completing her PhD degree she will return to Portugal to Universidade dos Açores, Departamento de Ciências Agrária, where she was teacher assistantship since 1995 and she will become assistant professor.

From now on she can be reached at: Universidade dos Açores

Departamento de Ciências Agrária

Terra-Chã

9700 Angra do Heroísmo

Terceira, Açores, Portugal

gsilveira@mail.angra.uac.pt

LIST OF PUBLICATIONS

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